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(54) Title: NON-TRANSGENIC HERBICIDE RESISTANT PLANTS

(57) Abstract: The present invention relates to the production of a non-transgenic plant resistant or tolerant to a herbicide of the phosphonomethylglycine family, e.g., glyphosate. The present invention also relates to the use of a recombinagenic Oligonucleobase to make a desired mutation in the chromosomal or episomal sequences of a plant in the gene encoding for 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS). The mutated protein, which substantially maintains the catalytic activity of the wild-type protein, allows for increased resistance or tolerance of the plant to a herbicide of the phosphonomethylglycine family, and allows for the substantially normal growth or development of the plant, its organs, tissues or cells as compared to the wild-type plant irrespective of the presence or absence of the herbicide. The present invention also relates to a non-transgenic plant cell in which the EPSPS gene has been mutated, a non-transgenic plant regenerated therefrom, as well as a plant resulting from a cross using a regenerated non-transgenic plant having a mutated EPSPS gene.

## **NON-TRANSGENIC HERBICIDE RESISTANT PLANTS**

The present application claims priority to U.S. Provisional Application No. 60/158,027, filed on October 7, 1999 and to U.S. Provisional Application No. 60/173,564, filed December 30, 1999, the disclosures of each of which are incorporated by reference herein in their entirety.

### **1. FIELD OF THE INVENTION**

The present invention relates to the production of a non-transgenic plant resistant or tolerant to a herbicide of the phosphonomethylglycine family, *e.g.*, glyphosate. The present invention also relates to the use of a recombinogenic oligonucleobase to make a desired mutation in the chromosomal or episomal sequences of a plant in the gene encoding for 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS). The mutated protein, which substantially maintains the catalytic activity of the wild-type protein, allows for increased resistance or tolerance of the plant to a herbicide of the phosphonomethylglycine family, and allows for the substantially normal growth or development of the plant, its organs, tissues or cells as compared to the wild-type plant irrespective of the presence or absence of the herbicide. The present invention also relates to a non-transgenic plant cell in which the EPSPS gene has been mutated, a non-transgenic plant regenerated therefrom, as well as a plant resulting from a cross using a regenerated non-transgenic plant having a mutated EPSPS gene.

### **2. BACKGROUND TO THE INVENTION**

#### **2.1 PHOSPHONOMETHYLGLYCINE HERBICIDES**

Herbicide-tolerant plants may reduce the need for tillage to control weeds thereby effectively reducing soil erosion. One herbicide which is the subject of much investigation in this regard is N-phosphonomethylglycine, commonly referred to as glyphosate. Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids, hormones and vitamins. Specifically, glyphosate curbs the conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (hereinafter referred to as EPSP synthase or EPSPS). For purposes of the present invention, the term "glyphosate" includes any herbicidally effective form of N-phosphonomethylglycine (including any salt thereof), other forms which result in the production of the glyphosate anion in plants and any other herbicides of the phosphonomethylglycine family.

Tolerance of plants to glyphosate can be increased by introducing a mutant EPSPS gene having an alteration in the EPSPS amino acid coding sequence into the genome of the plant. Examples of some of the mutations in the EPSPS gene for inducing glyphosate tolerance are described in the following patents: U.S. Patent No. 5,310,667; U.S. Patent No. 5,866,775; U.S. Patent No. 5,312,910; U.S. Patent No. 5,145,783. These proposed mutations typically have a higher  $K_i$  for glyphosate than the wild-type EPSPS enzyme which confers the glyphosate-tolerant phenotype, but these variants are also characterized by a high  $K_m$  for PEP which makes the enzyme kinetically less efficient (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663; Schulz et al., 1984, Arch. Microbiol. 137: 121-123; Sost et al., 1984, FEBS Lett. 173: 238-241; Kishore et al., 1986, Fed. Proc. 45: 1506; Sost and Amrhein, 1990, Arch. Biochem. Biophys. 282: 433-436). Many mutations of the EPSPS gene are chosen so as to produce an EPSPS enzyme that is resistant to herbicides, but unfortunately, the EPSPS enzyme produced by the mutated EPSPS gene has a significantly lower enzymatic activity than the wild-type EPSPS. For example, the apparent  $K_m$  for PEP and the apparent  $K_i$  for glyphosate for the wild-type EPSPS from *E. coli* are 10  $\mu$ M and 0.5  $\mu$ M, while for a glyphosate-tolerant isolate having a single amino acid substitution of alanine for glycine at position 96, these values are 220  $\mu$ M and 4.0 mM, respectively. A number of glyphosate-tolerant EPSPS genes have been constructed by mutagenesis. Again, the glyphosate-tolerant EPSPS had lower catalytic efficiency ( $V_{max}/K_m$ ), as shown by an increase in the  $K_m$  for PEP, and a slight reduction of the  $V_{max}$  of the wild-type plant enzyme (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663).

Since the kinetic constants of the variant enzymes are impaired with respect to PEP, it has been proposed that high levels of overproduction of the variant enzyme, 40-80 fold, would be required to maintain normal catalytic activity in plants in the presence of glyphosate (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663). It has been shown that glyphosate-tolerant plants can be produced by inserting into the genome of the plant the capacity to produce a higher level of EPSP synthase in the chloroplast of the cell (Shah et al., 1986, Science 233, 478-481), which enzyme is preferably glyphosate-tolerant (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663).

The introduction of the exogenous mutant EPSPS genes into plant is well documented. For example, according to U.S. Patent No. 4,545,060, to increase a plant's resistance to glyphosate, a gene coding for an EPSPS variant having at least one mutation that renders the enzyme more resistant to its competitive inhibitor, *i.e.*, glyphosate, is introduced into the plant genome. However, many complications and problems are associated with these examples. Many such mutations result in low expression of the mutated EPSPS gene product or result in an EPSPS gene product with significantly lower

enzymatic activity as compared to wild type. The low expression or low enzymatic activity of the mutated enzyme results in abnormally low levels of growth and development of the plant.

While such variants in the EPSP synthases have proved useful in obtaining transgenic plants tolerant to glyphosate, it would be increasingly beneficial to obtain a variant EPSPS gene product that is highly glyphosate-tolerant but still kinetically efficient, such that improved tolerance can be obtained with a wild-type expression level.

## 2.2 RECOMBINAGENIC OLIGONUCLEOBASES

Recombinagenic oligonucleobases and their use to effect genetic changes in eukaryotic cells are described in United States patent No. 5,565,350 to Kmiec (Kmiec I). Kmiec I teaches a method for introducing specific genetic alterations into a target gene. Kmiec I discloses, *inter alia*, recombinagenic oligonucleobases having two strands, in which a first strand contains two segments of at least 8 RNA-like nucleotides that are separated by a third segment of from 4 to about 50 DNA-like nucleotides, termed an "interposed DNA segment." The nucleotides of the first strand are base paired to DNA-like nucleotides of a second strand. The first and second strands are additionally linked by a segment of single stranded nucleotides so that the first and second strands are parts of a single oligonucleotide chain. Kmiec I further teaches a method for introducing specific genetic alterations into a target gene. According to Kmiec I, the sequences of the RNA segments are selected to be homologous, *i.e.*, identical, to the sequence of a first and a second fragment of the target gene. The sequence of the interposed DNA segment is homologous with the sequence of the target gene between the first and second fragment except for a region of difference, termed the "heterologous region." The heterologous region can effect an insertion or deletion, or can contain one or more bases that are mismatched with the sequence of target gene so as to effect a substitution. According to Kmiec I, the sequence of the target gene is altered as directed by the heterologous region, such that the target gene becomes homologous with the sequence of the recombinagenic oligonucleobase. Kmiec I specifically teaches that ribose and 2'-O-methylribose, *i.e.*, 2'-methoxyribose, containing nucleotides can be used in recombinagenic oligonucleobases and that naturally-occurring deoxyribose-containing nucleotides can be used as DNA-like nucleotides.

U.S. Patent No. 5,731,181 to Kmiec (Kmiec II) specifically disclose the use of recombinagenic oligonucleobases to effect genetic changes in plant cells and discloses further examples of analogs and derivatives of RNA-like and DNA-like nucleotides that can be used to effect genetic changes in specific target genes. Other patents discussing the use

of recombinagenic oligonucleobases include: U.S. Patent Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT/US00/23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

- 5 Recombinagenic oligonucleobases include mixed duplex oligonucleotides, non-nucleotide containing molecules taught in Kmiec II and other molecules taught in the above-noted patents and patent publications.

Citation or identification of any reference in Section 2, or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

### 3. SUMMARY OF THE INVENTION

The present invention is directed to a non-transgenic plant or plant cell having one or more mutations in the EPSPS gene, which plant has increased resistance or tolerance to a member of the phosphonomethylglycine family and which plant exhibits substantially normal growth or development of the plant, its organs, tissues or cells, as compared to the corresponding wild-type plant or cell. The present invention is also directed to a non-transgenic plant having a mutation in the EPSPS gene, which plant is resistant to or has an increased tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate, wherein the mutated EPSPS protein has substantially the same catalytic activity as compared to the wild-type EPSPS protein.

The present invention is also directed to a method for producing a non-transgenic plant having a mutated EPSPS gene that substantially maintains the catalytic activity of the wild-type protein irrespective of the presence or absence of a herbicide of the phosphonomethylglycine family. The method comprises introducing into a plant cell a recombinagenic oligonucleobase with a targeted mutation in the EPSPS gene and identifying a cell, seed, or plant having a mutated EPSPS gene.

Illustrative examples of a recombinagenic oligonucleobase is found in following patent publications, which are incorporated in their entirety by reference herein:

30 U.S. Patent Nos. 5,565,350; 5,756,325; 5,871,984; 5,760,012; 5,731,181; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT/US00/23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

The plant can be of any species of dicotyledonous, monocotyledonous or gymnospermous plant, including any woody plant species that grows as a tree or shrub, any herbaceous species, or any species that produces edible fruits, seeds or vegetables, or any

species that produces colorful or aromatic flowers. For example, the plant may be selected from a species of plant from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax, oilseed rape, cucumber, morning glory, balsam, pepper, eggplant, marigold, lotus, cabbage, daisy, carnation, tulip, iris, lily, and nut producing plants insofar as they are not already specifically mentioned.

The recombinagenic oligonucleobase can be introduced into a plant cell using any method commonly used in the art, including but not limited to, microcarriers (biolistic delivery), microfibers, electroporation, microinjection.

The invention is also directed to the culture of cells mutated according to the methods of the present invention in order to obtain a plant that produces seeds, henceforth a "fertile plant", and the production of seeds and additional plants from such a fertile plant.

The invention is further directed to a method of selectively controlling weeds in a field, the field comprising plants with the disclosed EPSPS gene alterations and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

The invention is also directed to novel mutations in the EPSPS gene that confer resistance or tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate, to a plant or wherein the mutated EPSPS has substantially the same enzymatic activity as compared to wild-type EPSPS.

### 3.1 DEFINITIONS

The invention is to be understood in accordance with the following definitions.

An oligonucleobase is a polymer of nucleobases, which polymer can hybridize by Watson-Crick base pairing to a DNA having the complementary sequence.

Nucleobases comprise a base, which is a purine, pyrimidine, or a derivative or analog thereof. Nucleobases include peptide nucleobases, the subunits of peptide nucleic acids, and morpholine nucleobases as well as nucleosides and nucleotides. Nucleosides are nucleobases that contain a pentosefuranosyl moiety, *e.g.*, an optionally substituted riboside or 2'-deoxyriboside. Nucleosides can be linked by one of several linkage moieties, which may or may not contain a phosphorus. Nucleosides that are linked by unsubstituted phosphodiester linkages are termed nucleotides.

An oligonucleobase chain has a single 5' and 3' terminus, which are the ultimate nucleobases of the polymer. A particular oligonucleobase chain can contain



nucleobases of all types. An oligonucleobase compound is a compound comprising one or more oligonucleobase chains that are complementary and hybridized by Watson-Crick base pairing. Nucleobases are either deoxyribo-type or ribo-type. Ribo-type nucleobases are pentosefuranosyl containing nucleobases wherein the 2' carbon is a methylene substituted  
5 with a hydroxyl, alkyloxy or halogen. Deoxyribo-type nucleobases are nucleobases other than ribo-type nucleobases and include all nucleobases that do not contain a pentosefuranosyl moiety.

An oligonucleobase strand generically includes both oligonucleobase chains and segments or regions of oligonucleobase chains. An oligonucleobase strand has a 3' end  
10 and a 5' end. When a oligonucleobase strand is coextensive with a chain, the 3' and 5' ends of the strand are also 3' and 5' termini of the chain.

According to the present invention, substantially normal growth of a plant, plant organ, plant tissue or plant cell is defined as a growth rate or rate of cell division of the plant, plant organ, plant tissue, or plant cell that is at least 35%, at least 50%, at least 60%,  
15 or at least 75% of the growth rate or rate of cell division in a corresponding plant, plant organ, plant tissue or plant cell expressing the wild type EPSPS protein.

According to the present invention, substantially normal development of a plant, plant organ, plant tissue or plant cell is defined as the occurrence of one or more developmental events in the plant, plant organ, plant tissue or plant cell that are  
20 substantially the same as those occurring in a corresponding plant, plant organ, plant tissue or plant cell expressing the wild type EPSPS protein.

According to the present invention plant organs include, but are not limited to, leaves, stems, roots, vegetative buds, floral buds, meristems, embryos, cotyledons, endosperm, sepals, petals, pistils, carpels, stamens, anthers, microspores, pollen, pollen  
25 tubes, ovules, ovaries and fruits, or sections, slices or discs taken therefrom. Plant tissues include, but are not limited to, callus tissues, ground tissues, vascular tissues, storage tissues, meristematic tissues, leaf tissues, shoot tissues, root tissues, gall tissues, plant tumor tissues, and reproductive tissues. Plant cells include, but are not limited to, isolated cells with cell walls, variously sized aggregates thereof, and protoplasts.

30 Plants are substantially "tolerant" to glyphosate when they are subjected to it and provide a dose/response curve which is shifted to the right when compared with that provided by similarly subjected non-tolerant like plant. Such dose/response curves have "dose" plotted on the X-axis and "percentage kill", "herbicidal effect", etc., plotted on the y-axis. Tolerant plants will require more herbicide than non-tolerant like plants in order to  
35 produce a given herbicidal effect. Plants which are substantially "resistant" to the glyphosate exhibit few, if any, necrotic, lytic, chlorotic or other lesions, when subjected to

glyphosate at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Plants which are resistant to a herbicide are also tolerant of the herbicide. The terms "resistant" and "tolerant" are to be construed as "tolerant and/or resistant" within the context of the present application.

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#### 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is the DNA sequence of *Arabidopsis thaliana* EPSPS gene (SEQ ID NO:1). The bold underlined nucleotide residues are the targeted residues.

FIG. 1B is the amino acid sequence of *Arabidopsis thaliana* EPSPS protein  
10 (SEQ ID NO:2). The bold and underlined amino acid residues are the targeted residues.

FIG. 2 is a list of the *Arabidopsis thaliana* wild-type and mutant EPSPS nucleotide and amino acid sequences in the region of amino acid position 173 to 183; wild-type nucleotide sequence (SEQ ID NO:1) and wild-type amino acid sequence (SEQ ID NO:2); mutant A<sub>177</sub> nucleotide sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4); mutant I<sub>178</sub> nucleotide sequence (SEQ ID NO:5) and amino acid sequence (SEQ ID NO:6); mutant A<sub>177</sub>I<sub>178</sub> nucleotide sequence (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8); mutant I<sub>178</sub>S<sub>182</sub> nucleotide sequence (SEQ ID NO:9) and amino acid sequence (SEQ ID NO:10); mutant A<sub>177</sub>S<sub>182</sub> nucleotide sequence (SEQ ID NO:11) and amino acid sequence (SEQ ID NO:12); mutant A<sub>177</sub>I<sub>178</sub>S<sub>182</sub> nucleotide sequence (SEQ ID NO:13) and  
20 amino acid sequence (SEQ ID NO:14); mutant V<sub>177</sub>S<sub>182</sub> nucleotide sequence (SEQ ID NO:15) and amino acid sequence (SEQ ID NO:16); mutant L<sub>178</sub>S<sub>182</sub> nucleotide sequence (SEQ ID NO:17) and amino acid sequence (SEQ ID NO:18); mutant A<sub>177</sub>V<sub>178</sub> nucleotide sequence (SEQ ID NO:19) and amino acid sequence (SEQ ID NO:20); mutant A<sub>177</sub>L<sub>182</sub> nucleotide sequence (SEQ ID NO:21) and amino acid sequence (SEQ ID NO:22).

FIG. 3A-C is an alignment of the DNA of *Arabidopsis thaliana* EPSPS gene performed by DNASTar (LaserGene), (SEQ ID NO:1) with the nucleotide sequences of *Brassica napus* (SEQ ID NO:23); *Petunia hybrida* (SEQ ID NO:24); and *Zea mays* (SEQ ID NO:25) EPSPS gene. The sequences are aligned using J. Hein method with weighted residue weight table.

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FIG. 4 is an alignment of the *Arabidopsis thaliana* EPSPS amino acid sequence (SEQ ID NO:2) with the *Brassica napus* (SEQ ID NO:26); *Petunia hybrida* (SEQ ID NO:27); and *Zea mays* (SEQ ID NO:28) EPSPS amino acid sequences. The sequences are aligned using J. Hein method with weighted residue weight table.

FIG. 5 is a list of the mutagenesis primers used, with the targeted codons in bold characters (mutant primer A<sub>177</sub> (SEQ ID NO:29); mutant primer I<sub>178</sub> (SEQ ID NO:30);

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mutant primer A<sub>177</sub>I<sub>178</sub> (SEQ ID NO:31); mutant primer I<sub>178</sub>S<sub>182</sub> (SEQ ID NO:32); mutant primer A<sub>177</sub>S<sub>182</sub> (SEQ ID NO:34); mutant primer A<sub>177</sub>I<sub>178</sub>S<sub>182</sub> (SEQ ID NO:35); mutant primer V<sub>177</sub>S<sub>182</sub> (SEQ ID NO:35); mutant primer L<sub>178</sub>S<sub>182</sub> (SEQ ID NO:36); mutant primer A<sub>177</sub>V<sub>178</sub> (SEQ ID NO:37); and mutant primer A<sub>177</sub>L<sub>182</sub> (SEQ ID NO:38)).

5 FIG. 6 is the growth measured by optical density at 600 nm of *Arabidopsis* clones in the presence (+) and absence (-) of 17 mM glyphosate.

FIG. 7 (top panel) is a western blot showing the expression of His-tagged *Bacillus*, *Arabidopsis* wild type (WT) and mutant (AS) EPSPS proteins isolated from cell lysates (L) and eluates (E). Untransformed *Salmonella* as a negative control shows no  
10 EPSPS expression. The bottom panel is a silver-stained duplicate gel.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a non-transgenic plant or plant cell having a mutation in the EPSPS gene, which plant has increased resistance or tolerance to a  
15 member of the phosphonomethylglycine family and which plant exhibits substantially normal growth or development of the plant, its organs, tissues or cells, as compared to the corresponding wild-type plant or cell. The present invention is also directed to a non-transgenic plant having a mutation in the EPSPS gene, which plant is resistant to or has an increased tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate,  
20 wherein the mutated EPSPS protein has substantially the same catalytic activity as compared to the wild-type EPSPS protein.

The present invention is also directed to a method for producing a non-transgenic plant having a mutated EPSPS gene that substantially maintains the catalytic activity of the wild-type protein irrespective of the presence or absence of a herbicide of the  
25 phosphonomethylglycine family. The method comprises introducing into a plant cell a recombinogenic oligonucleobase with a targeted mutation in the EPSPS gene and identifying a cell, seed, or plant having a mutated EPSPS gene.

Illustrative examples of a recombinogenic oligonucleobase is found in following patent publications, which are incorporated in their entirety by reference herein:  
30 U.S. Patent Nos. 5,565,350; 5,756,325; 5,871,984; 5,760,012; 5,731,181; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT/US00/23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

The plant can be of any species of dicotyledonous, monocotyledonous or  
35 gymnospermous plant, including any woody plant species that grows as a tree or shrub, any herbaceous species, or any species that produces edible fruits, seeds or vegetables, or any

species that produces colorful or aromatic flowers. For example, the plant may be selected from a species of plant from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, onion, soya spp, sugar cane, pea, field beans, poplar, 5 grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax, oilseed rape, cucumber, morning glory, balsam, pepper, eggplant, marigold, lotus, cabbage, daisy, carnation, tulip, iris, lily, and nut producing plants insofar as they are not already specifically mentioned.

The recombinagenic oligonucleobase can be introduced into a plant cell using any method commonly used in the art, including but not limited to, microcarriers 10 (biolistic delivery), microfibers, electroporation, microinjection.

The invention is also directed to the culture of cells mutated according to the methods of the present invention in order to obtain a plant that produces seeds, henceforth a "fertile plant", and the production of seeds and additional plants from such a fertile plant.

The invention is further directed to a method of selectively controlling weeds 15 in a field, the field comprising plants with the disclosed EPSPS gene alterations and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

The invention is also directed to novel mutations in the EPSPS gene that confer resistance or tolerance to a member of the phosphonomethylglycine family, *e.g.*, 20 glyphosate, to a plant or wherein the mutated EPSPS has substantially the same enzymatic activity as compared to wild-type EPSPS.

### 5.1 RECOMBINAGENIC OLIGONUCLEOBASES

The invention can be practiced with recombinagenic oligonucleobases 25 having the conformations and chemistries described in United States patent No. 5,565,350 to Kmiec (Kmiec I) and U.S. patent No. 5,731,181 (Kmiec II) gene, which are hereby incorporated by reference. Kmiec I teaches a method for introducing specific genetic alterations into a target gene. The recombinagenic oligonucleobases in Kmiec I and/or Kmiec II contain two complementary strands, one of which contains at least one segment of 30 RNA-type nucleotides (an "RNA segment") that are base paired to DNA-type nucleotides of the other strand.

Kmiec II discloses that purine and pyrimidine base-containing non-nucleotides can be substituted for nucleotides. U.S. Patent Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in 35 International Patent No. PCT/US00/23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789, which are each

hereby incorporated in their entirety, disclose additional recombinagenic molecules that can be used for the present invention. The term "recombinagenic oligonucleobase" is used herein to denote the molecules that can be used in the methods of the present invention and include mixed duplex oligonucleotides, non-nucleotide containing molecules taught in  
5 Kmiec II, single stranded oligodeoxynucleotides and other recombinagenic molecules taught in the above noted patents and patent publications.

In one embodiment, the recombinagenic oligonucleobase is a mixed duplex oligonucleotide in which the RNA-type nucleotides of the mixed duplex oligonucleotide are made RNase resistant by replacing the 2'-hydroxyl with a fluoro, chloro or bromo  
10 functionality or by placing a substituent on the 2'-O. Suitable substituents include the substituents taught by the Kmiec II. Alternative substituents include the substituents taught by U.S. Patent No. 5,334,711 (Sproat) and the substituents taught by patent publications EP 629 387 and EP 679 657 (collectively, the Martin Applications), which are hereby incorporated by reference. As used herein, a 2' -fluoro, chloro or bromo derivative of a  
15 ribonucleotide or a ribonucleotide having a 2'-OH substituted with a substituent described in the Martin Applications or Sproat is termed a "2'-Substituted Ribonucleotide." As used herein the term "RNA-type nucleotide" means a 2'-hydroxyl or 2'-Substituted Nucleotide that is linked to other nucleotides of a mixed duplex oligonucleotide by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II.  
20 As used herein the term "deoxyribo-type nucleotide" means a nucleotide having a 2'-H, which can be linked to other nucleotides of a MDON by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II.

In a particular embodiment of the present invention, the recombinagenic oligonucleobase is a mixed duplex oligonucleotide that is linked solely by unsubstituted  
25 phosphodiester bonds. In alternative embodiments, the linkage is by substituted phosphodiester, phosphodiester derivatives and non-phosphorus-based linkages as taught by Kmiec II. In yet another embodiment, each RNA-type nucleotide in the mixed duplex oligonucleotide is a 2'-Substituted Nucleotide. Particular preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-  
30 hydroxyethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. More preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides. In another embodiment the mixed duplex oligonucleotide is linked by unsubstituted phosphodiester bonds.

35 Although mixed duplex oligonucleotide having only a single type of 2'-substituted RNA-type nucleotide are more conveniently synthesized, the methods of the

invention can be practiced with mixed duplex oligonucleotides having two or more types of RNA-type nucleotides. The function of an RNA segment may not be affected by an interruption caused by the introduction of a deoxynucleotide between two RNA-type trinucleotides, accordingly, the term RNA segment encompasses such an "interrupted RNA segment." An uninterrupted RNA segment is termed a contiguous RNA segment. In an alternative embodiment an RNA segment can contain alternating RNase-resistant and unsubstituted 2'-OH nucleotides. The mixed duplex oligonucleotides preferably have fewer than 100 nucleotides and more preferably fewer than 85 nucleotides, but more than 50 nucleotides. The first and second strands are Watson-Crick base paired. In one embodiment the strands of the mixed duplex oligonucleotide are covalently bonded by a linker, such as a single stranded hexa, penta or tetranucleotide so that the first and second strands are segments of a single oligonucleotide chain having a single 3' and a single 5' end. The 3' and 5' ends can be protected by the addition of a "hairpin cap" whereby the 3' and 5' terminal nucleotides are Watson-Crick paired to adjacent nucleotides. A second hairpin cap can, additionally, be placed at the junction between the first and second strands distant from the 3' and 5' ends, so that the Watson-Crick pairing between the first and second strands is stabilized.

The first and second strands contain two regions that are homologous with two fragments of the target EPSPS gene, *i.e.*, have the same sequence as the target gene. A homologous region contains the nucleotides of an RNA segment and may contain one or more DNA-type nucleotides of connecting DNA segment and may also contain DNA-type nucleotides that are not within the intervening DNA segment. The two regions of homology are separated by, and each is adjacent to, a region having a sequence that differs from the sequence of the target gene, termed a "heterologous region." The heterologous region can contain one, two or three mismatched nucleotides. The mismatched nucleotides can be contiguous or alternatively can be separated by one or two nucleotides that are homologous with the target gene. Alternatively, the heterologous region can also contain an insertion or one, two, three or of five or fewer nucleotides. Alternatively, the sequence of the mixed duplex oligonucleotide may differ from the sequence of the target gene only by the deletion of one, two, three, or five or fewer nucleotides from the mixed duplex oligonucleotide. The length and position of the heterologous region is, in this case, deemed to be the length of the deletion, even though no nucleotides of the mixed duplex oligonucleotide are within the heterologous region. The distance between the fragments of the target gene that are complementary to the two homologous regions is identically the length of the heterologous region when a substitution or substitutions is intended. When the heterologous region contains an insertion, the homologous regions are thereby separated in

the mixed duplex oligonucleotide farther than their complementary homologous fragments are in the gene, and the converse is applicable when the heterologous region encodes a deletion.

The RNA segments of the mixed duplex oligonucleotides are each a part of a homologous region, *i.e.*, a region that is identical in sequence to a fragment of the target gene, which segments together preferably contain at least 13 RNA-type nucleotides and preferably from 16 to 25 RNA-type nucleotides or yet more preferably 18-22 RNA-type nucleotides or most preferably 20 nucleotides. In one embodiment, RNA segments of the homology regions are separated by and adjacent to, *i.e.*, "connected by" an intervening DNA segment. In one embodiment, each nucleotide of the heterologous region is a nucleotide of the intervening DNA segment. An intervening DNA segment that contains the heterologous region of a mixed duplex oligonucleotide is termed a "mutator segment."

The change to be introduced into the target EPSPS gene is encoded by the heterologous region. The change to be introduced into the EPSPS gene may be a change in one or more bases of the EPSPS gene sequence or the addition or deletion of one or more bases.

In another embodiment of the present invention, the recombinagenic oligonucleobase is a single stranded oligodeoxynucleotide mutational vector or SSOMV, which is disclosed in International Patent Application PCT/US00/23457, which is incorporated by reference in its entirety. The sequence of the SSOMV is based on the same principles as the mutational vectors described in U.S. Patent Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789. The sequence of the SSOMV contains two regions that are homologous with the target sequence separated by a region that contains the desired genetic alteration termed the mutator region. The mutator region can have a sequence that is the same length as the sequence that separates the homologous regions in the target sequence, but having a different sequence. Such a mutator region can cause a substitution. Alternatively, the homologous regions in the SSOMV can be contiguous to each other, while the regions in the target gene having the same sequence are separated by one, two or more nucleotides. Such a SSOMV causes a deletion from the target gene of the nucleotides that are absent from the SSOMV. Lastly, the sequence of the target gene that is identical to the homologous regions may be adjacent in the target gene but separated by one two or more nucleotides in the sequence of the SSOMV. Such an SSOMV causes an insertion in the sequence of target gene.

The nucleotides of the SSOMV are deoxyribonucleotides that are linked by unmodified phosphodiester bonds except that the 3' terminal and/or 5' terminal internucleotide linkage or alternatively the two 3' terminal and/or 5' terminal internucleotide linkages can be a phosphorothioate or phosphoamidate. As used herein an internucleotide linkage is the linkage between nucleotides of the SSOMV and does not include the linkage between the 3' end nucleotide or 5' end nucleotide and a blocking substituent, see *supra*. In a specific embodiment the length of the SSOMV is between 21 and 55 deoxynucleotides and the lengths of the homology regions are, accordingly, a total length of at least 20 deoxynucleotides and at least two homology regions should each have lengths of at least 8 deoxynucleotides.

The SSOMV can be designed to be complementary to either the coding or the non-coding strand of the target gene. When the desired mutation is a substitution of a single base, it is preferred that both the mutator nucleotide be a pyrimidine. To the extent that is consistent with achieving the desired functional result it is preferred that both the mutator nucleotide and the targeted nucleotide in the complementary strand be pyrimidines. Particularly preferred are SSOMV that encode transversion mutations, *i.e.*, a C or T mutator nucleotide is mismatched, respectively, with a C or T nucleotide in the complementary strand.

In addition to the oligodeoxynucleotide the SSOMV can contain a 5' blocking substituent that is attached to the 5' terminal carbons through a linker. The chemistry of the linker is not critical other than its length, which should preferably be at least 6 atoms long and that the linker should be flexible. A variety of non-toxic substituents such as biotin, cholesterol or other steroids or a non-intercalating cationic fluorescent dye can be used. Particularly preferred as reagents to make SSOMV are the reagents sold as Cy3<sup>TM</sup> and Cy5<sup>TM</sup> by Glen Research, Sterling VA, which are blocked phosphoroamidites that upon incorporation into an oligonucleotide yield 3,3,3',3'-tetramethyl N,N'-isopropyl substituted indomonocarbocyanine and indodicarbocyanine dyes, respectively. Cy3 is the most preferred. When the indocarbocyanine is N-oxyalkyl substituted it can be conveniently linked to the 5' terminal of the oligodeoxynucleotide through as a phosphodiester with a 5' terminal phosphate. The chemistry of the dye linker between the dye and the oligodeoxynucleotide is not critical and is chosen for synthetic convenience. When the commercially available Cy3 phosphoramidite is used as directed the resulting 5' modification consists of a blocking substituent and linker together which are a N-hydroxypropyl, N'-phosphatidylpropyl 3,3,3',3'-tetramethyl indomonocarbocyanine.

In the preferred embodiment the indocarbocyanine dye is tetra substituted at the 3 and 3' positions of the indole rings. Without limitation as to theory these substitutions



prevent the dye from being an intercalating dye. The identity of the substituents at these positions are not critical. The SSOMV can in addition have a 3' blocking substituent. Again the chemistry of the 3' blocking substituent is not critical.

## 5 5.2 THE LOCATION AND TYPE OF MUTATION INTRODUCED INTO THE EPSPS GENE

In one embodiment of the present invention, the *Arabidopsis thaliana* EPSPS gene (see Figure 1A) and corresponding EPSPS enzyme (see Figure 1B) comprises a mutation at one or more amino acid residues selected from the group consisting of Leu<sub>173</sub>,  
10 Gly<sub>177</sub>, Thr<sub>178</sub>, Ala<sub>179</sub>, Met<sub>180</sub>, Arg<sub>181</sub>, Pro<sub>182</sub>, Ser<sub>98</sub>, Ser<sub>255</sub> and Leu<sub>198</sub>, or at an analogous position in an EPSPS paralog, and the mutation results in one or more of the following amino acid substitutions in the EPSPS enzyme in comparison with the wild-type sequence:

- (i) Leu<sub>173</sub> - Phe
- (ii) Gly<sub>177</sub> - Ala or Ile
- 15 (iii) Thr<sub>178</sub> - Ile or Val or Leu
- (iv) Ala<sub>179</sub> - Gly
- (v) Met<sub>180</sub> - Cys
- (vi) Arg<sub>181</sub> - Leu or Ser
- (vii) Pro<sub>182</sub> - Leu or Ser
- 20 (viii) Ser<sub>98</sub> - Asp
- (ix) Ser<sub>255</sub> - Ala
- (x) Leu<sub>198</sub> - Lys.

In another embodiment of the present invention, within the EPSPS gene product, the amino acid residue to be changed is Leu within the contiguous sequence Leu-  
25 Tyr-Leu-Gly-Asn (SEQ ID NO:29) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg  
30 (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to  
35 Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the

contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

5 Alternatively, and/or additionally, the mutation may result in the replacement of any amino acid at positions corresponding to 256, 284-288 and 353-356 with respect to the EPSPS protein depicted in Figure 1B (SEQ ID NO. 2).

In specific embodiments of the present invention, the EPSPS gene is mutated at amino acid position 177 in which Gly is replaced by Ala. Another specific embodiment  
10 is the substitution of Thr at amino acid position 178 by Ile. A further specific embodiment comprises a mutation at amino acid position 177 in which Gly is replaced by Ala, plus the additional substitution of Thr at amino acid position 178 by Ile. Other specific embodiments of the present invention are directed to mutations at amino acid position 178, in which Thr is replaced by Ile, plus the additional mutation at position 182, in which Pro is  
15 replaced by Ser. Other embodiments include the substitution of Gly at amino acid position 177 by Ala, plus the additional mutation at amino acid position 182, in which Pro is substituted by Ser. Other mutated EPSPS sequences comprise the substitution of Gly at position 177 by Ala, plus the substitution at position 178, in which Thr is replaced by Ile, plus the additional substitution of Pro at amino acid position 182 by Ser. Another  
20 embodiment is the substitution of Thr at amino acid position 178 by Val and the additional mutation at amino acid position 182, in which Pro is replaced by Ser. A further specific embodiment includes the substitution of Thr at position 178 by Leu, plus the mutation at amino acid position 182, in which Pro is replaced by Ser. A further embodiment includes, the substitution at amino acid position 177 in which Gly is replaced by Ala, plus the  
25 substitution of Thr at position 178 by Val. The invention also embodies the substitution at amino acid position 177 in which Gly is replaced by Ala, plus the replacement of Thr at amino acid position 178 by Leu (see Figure 2).

The foregoing mutations in the EPSPS gene were described using the *Arabidopsis thaliana* EPSPS gene (SEQ ID NO:1) and protein (SEQ ID NO:2). The  
30 present invention also encompasses mutant EPSPS genes of other species (paralogs). However, due to variations in the EPSPS genes of different species, the number of the amino acid residue to be changed in one species may be different in another species. Nevertheless, the analogous position is readily identified by one of skill in the art by sequence homology. For example, Figure 3A-C shows the aligned nucleotide sequences  
35 and Figure 4 shows the aligned amino acid sequences of four paralogs of the EPSPS gene, *Arabidopsis thaliana*, *Zea mays*, *Petunia hybrida*, and *Brassica napus*. Thus, the analogous

positions in *Zea mays* are Leu<sub>97</sub>, Gly<sub>101</sub>, Thr<sub>102</sub>, Ala<sub>103</sub>, Met<sub>104</sub>, Arg<sub>105</sub>, Pro<sub>106</sub>, Ser<sub>23</sub>, Ser<sub>179</sub> and Leu<sub>122</sub>. Thus, the *Zea mays* EPSPS amino acid sequence is mutated at one or more of the following amino acid positions and results in one or more of the following substitutions:

- (i) Leu<sub>97</sub> - Phe
- 5 (ii) Gly<sub>101</sub> - Ala or Ile
- (iii) Thr<sub>102</sub> - Ile or Val or Leu
- (iv) Ala<sub>103</sub> - Gly
- (v) Met<sub>104</sub> - Cys
- (vi) Arg<sub>105</sub> - Leu or Ser
- 10 (vii) Pro<sub>106</sub> - Leu or Ser
- (viii) Ser<sub>23</sub> - Asp
- (ix) Ser<sub>179</sub> - Ala
- (x) Leu<sub>122</sub> - Lys.

In another embodiment of the present invention, within the *Zea mays* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Phe-Leu-Gly-Asn (SEQ ID NO:39) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

In *Brassica napus*, the analogous amino acid positions are Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>, Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>. Thus, the *Brassica napus* EPSPS amino acid sequence is mutated at one or more of the following amino acid positions and results in one or more of the following substitutions:

- (i) Leu<sub>169</sub> - Phe

- (ii) Gly<sub>173</sub> - Ala or Ile
- (iii) Thr<sub>174</sub> - Ile or Val or Leu
- (iv) Ala<sub>175</sub> - Gly
- (v) Met<sub>176</sub> - Cys
- 5 (vi) Arg<sub>177</sub> - Leu or Ser
- (vii) Pro<sub>178</sub> - Leu or Ser
- (viii) Ser<sub>94</sub> - Asp
- (ix) Ser<sub>251</sub> - Ala
- (x) Leu<sub>194</sub> - Lys

10 In another embodiment of the present invention, within the *Brassica napus* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Tyr-Leu-Gly-Asn (SEQ ID NO:29) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the  
 15 contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-  
 20 Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the  
 25 amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

In *Petunia hybrida* the analogous positions are Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>, Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>. Thus, the *Petunia hybrida* EPSPS amino acid  
 30 sequence is mutated at one or more of the following amino acid positions and results in one or more of the following substitutions:

- (i) Leu<sub>169</sub> - Phe
- (ii) Gly<sub>173</sub> - Ala or Ile
- (iii) Thr<sub>174</sub> - Ile or Val or Leu
- 35 (iv) Ala<sub>175</sub> - Gly
- (v) Met<sub>176</sub> - Cys

- (vi) Arg<sub>177</sub> - Leu or Ser
- (vii) Pro<sub>178</sub> - Leu or Ser
- (viii) Ser<sub>94</sub> -Asp
- (ix) Ser<sub>251</sub> -Ala
- 5 (x) Leu<sub>194</sub> -Lys

In another embodiment of the present invention, within the *Petunia hybrida* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Phe-Leu-Gly-Asn (SEQ ID NO:39) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

### 25 5.3 THE DELIVERY OF RECOMBINAGENIC OLIGONUCLEOBASES INTO PLANT CELLS

Any commonly known method can be used in the methods of the present invention to transform a plant cell with a recombinagenic oligonucleobases. Illustrative methods are listed below.

30

#### 5.3.1 MICROCARRIERS AND MICROFIBERS

The use of metallic microcarriers (microspheres) for introducing large fragments of DNA into plant cells having cellulose cell walls by projectile penetration is well known to those skilled in the relevant art (henceforth biolistic delivery). United States Patent Nos. 4,945,050; 5,100,792 and 5,204,253 describe general techniques for selecting microcarriers and devices for projecting them.

35

Specific conditions for using microcarriers in the methods of the present invention are described in International Publication WO 99/07865. In an illustrative technique, ice cold microcarriers (60 mg/ml), mixed duplex oligonucleotide (60 mg/ml) 2.5 M  $\text{CaCl}_2$  and 0.1 M spermidine are added in that order; the mixture gently agitated, *e.g.*, by vortexing, for 10 minutes and let stand at room temperature for 10 minutes, whereupon the microcarriers are diluted in 5 volumes of ethanol, centrifuged and resuspended in 100% ethanol. Good results can be obtained with a concentration in the adhering solution of 8-10  $\mu\text{g}/\mu\text{l}$  microcarriers, 14-17  $\mu\text{g}/\text{ml}$  mixed duplex oligonucleotide, 1.1-1.4 M  $\text{CaCl}_2$  and 18-22 mM spermidine. Optimal results were observed under the conditions of 8  $\mu\text{g}/\mu\text{l}$  microcarriers, 16.5  $\mu\text{g}/\text{ml}$  mixed duplex oligonucleotide, 1.3 M  $\text{CaCl}_2$  and 21 mM spermidine.

Recombinagenic oligonucleobases can also be introduced into plant cells for the practice of the present invention using microfibers to penetrate the cell wall and cell membrane. U.S. Patent No. 5,302,523 to Coffee et al. describes the use of 30 x 0.5  $\mu\text{m}$  and 10 x 0.3  $\mu\text{m}$  silicon carbide fibers to facilitate transformation of suspension maize cultures of Black Mexican Sweet. Any mechanical technique that can be used to introduce DNA for transformation of a plant cell using microfibers can be used to deliver recombinagenic oligonucleobases for transmutation.

An illustrative technique for microfiber delivery of a recombinagenic oligonucleobase is as follows: Sterile microfibers (2  $\mu\text{g}$ ) are suspended in 150  $\mu\text{l}$  of plant culture medium containing about 10  $\mu\text{g}$  of a mixed duplex oligonucleotide. A suspension culture is allowed to settle and equal volumes of packed cells and the sterile fiber/nucleotide suspension are vortexed for 10 minutes and plated. Selective media are applied immediately or with a delay of up to about 120 hours as is appropriate for the particular trait.

### 5.3.2 PROTOPLAST ELECTROPORATION

In an alternative embodiment, the recombinagenic oligonucleobases can be delivered to the plant cell by electroporation of a protoplast derived from a plant part. The protoplasts are formed by enzymatic treatment of a plant part, particularly a leaf, according to techniques well known to those skilled in the art. *See, e.g.*, Gallois et al., 1996, in *Methods in Molecular Biology* 55:89-107, Humana Press, Totowa, NJ; Kipp et al., 1999, in *Methods in Molecular Biology* 133:213-221, Humana Press, Totowa, NJ. The protoplasts need not be cultured in growth media prior to electroporation. Illustrative conditions for electroporation are  $3 \times 10^5$  protoplasts in a total volume of 0.3 ml with a concentration of recombinagenic oligonucleobase of between 0.6 - 4  $\mu\text{g}/\text{mL}$ .

### 5.3.3 WHISKERS AND MICROINJECTION

In yet another alternative embodiment, the recombinagenic oligonucleobase can be delivered to the plant cell by whiskers or microinjection of the plant cell. The so called whiskers technique is performed essentially as described in Frame et al., 1994, Plant J. 6:941-948. The recombinagenic oligonucleobase is added to the whiskers and used to transform the plant cells. The recombinagenic oligonucleobase may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalyzed between the oligonucleotide and the target sequence in the EPSPS gene.

10

### 5.4 SELECTION OF GLYPHOSATE RESISTANT PLANTS

Plants or plant cells can be tested for resistance or tolerance to a herbicide using commonly known methods in the art, e.g., by growing the plant or plant cell in the presence of a herbicide and measuring the rate of growth as compared to the growth rate in the absence of the herbicide.

15

## 6. EXAMPLE

The following experiments demonstrate the production of mutant *Arabidopsis thaliana* EPSPS genes which are resistant to the herbicide glyphosate and which allows the plant cells to maintain a growth rate

20

### 6.1 MATERIAL AND METHODS

#### 6.1.1 ISOLATION OF ARABIDOPSIS THALIANA EPSPS cDNA

A 1.3 kb DNA fragment was amplified by PCR from an *Arabidopsis* cDNA library using the primers AtEXPEXPM1 and AtEXPEXP2CM-2. The two primers were designed to amplify the cDNA from the mature peptide to the termination codon. The 5' primer AtEXPEXPM1 contains an XbaI site (underlined) and the 3' primer AtEXPEXP2CM-2 contains a BglII site (underlined), sites which will be of use for cloning of the fragment into the expression vector.

30

AtEXPEXPM1

5'-GCTCTAGAGAAAGCGTCGGAGATTGTACTT-3' (SEQ ID NO:40)

AtEXPEXP2CM-2

35 5'-GCAGATCTGAGCTCTTAGTGCTTTGTGATTCTTTCAAGTAC-3' (SEQ ID NO:41)

The PCR band was excised from the agarose gel and purified (GeneClean, Biol). Its sequence was then confirmed as the mature peptide sequence of *Arabidopsis thaliana* EPSPS gene.

### 5 6.1.2 PREPARATION OF THE EXPRESSION VECTOR

The EPSPS coding region of the *AroE Bacillus subtilis* gene was obtained by PCR using the following primers:

BsAroE5'Xba

5'-GCGTCTAGAAAAACGAGATAAGGTGCAG-3' (SEQ ID NO:42) and

10

BsAroE3'BamHI

5'-GCGGATCCTCAGGATTTTTCGAAAGCTTATTTAAATG-3' (SEQ ID NO:43).

15 The PCR fragment, lacking an initiation codon (ATG), was cloned in-frame to the pACLacIMH6RecA vector by replacing the ORF of *RecA* by digesting with XbaI and BamHI. PACLacIMH6RecA contained the LacI region of Pet21 at positions 1440 to 3176, the MH6 RecA at positions 3809 to 5188, chloramphenicol resistance gene at positions 5445-218 (5446 to 5885 and 1 to 218), and the p15A origin of replication at positions 581  
20 to 1424. The coding region of *RecA* gene was cloned from *E.coli* in-frame with the start codon and 6 histidine linker (MH6) behind the LacZ promoter of pUC19.

### 6.1.3 CLONING OF THE ARABIDOPSIS EPSPS GENE INTO BACTERIAL EXPRESSION VECTOR

25 The *Arabidopsis* 1.3 kb PCR fragment was digested with XbaI and BamHI (compatible with BglII) and cloned into the plasmid pACYCLacIMH6EPSPS, in place of the *Bacillus* gene.

The clones obtained (selected on chloramphenicol) were then sequenced and confirmed positive. One of the confirmed clones (pAtEPS-12) was selected and the  
30 junctions between the cDNA and the cloning plasmid were also confirmed to be identical to the expected sequences.

#### 6.1.4 NOVEL POINT MUTATIONS IN THE EPSPS GENE

Ten different mutants of the *Arabidopsis thaliana* EPSPS gene were  
35 designed, (see Figure 2). For the mutagenesis experiments, PCR primers were designed  
with one, two or three mutations. The PCR reactions were performed using a regular



flanking primer (5'ATEPS-198: 5'- GAAAGCGTCGGAGATTGTAC-3' (SEQ ID NO:44)) and one of the mutation-carrying primers (see Figure 5).

The 353bp PCR fragments obtained were purified (Qiagen PCR Purification kit) and their sequence confirmed. The fragments were then digested with PstI (underlined in the primer sequences) and BamHI and ligated to the pAtEPS-12 vector, which had itself been previously digested with PstI and BamHI. JM109 (Promega) competent cells were used for the transformation and plated onto chloramphenicol-containing LB plates. Clones from each mutagenesis experiment were then isolated and their sequence confirmed.

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#### 6.1.5 GLYPHOSATE RESISTANCE ASSAYS

Electrocompetent cells of SA4247, a LacZ - *Salmonella typhi* strain, were prepared according to well known procedures (see Current Protocols in Molecular Biology, (Wiley and Sons, Inc.)). 30  $\mu$ l of SA4247 competent cells were electroporated with 20 ng of each plasmid DNA encoding *Arabidopsis* wild-type and mutant EPSPS proteins, *Bacillus* wild-type EPSPS, along with a mock transfection as a control. The settings for electroporation were 25  $\mu$ F, 2.5KV and 200 ohms. After electroporation, the cells were transferred into 15 mls culture tube and supplemented with 970  $\mu$ l of SOC medium. The cultures were incubated for 1 ½ hours at 37°C at 225 rpm. 50  $\mu$ l of each culture were plated onto LB plates containing 17  $\mu$ g/ml chloramphenicol (in duplicates) and incubated overnight at 37°C. On the following day, 5 colonies of each plate were picked and transferred onto M9 plates and incubated overnight at 37°C.

Colonies from the overnight incubation on solid M9 were inoculated into 4 ml of liquid M9 medium and grown overnight at 37°C. On the following day, 25 ml of liquid M9 medium containing chloramphenicol, IPTG and 17 mM or 0 mM Glyphosate (Aldrich, 33775-7) were inoculated with 1-2 mls of each overnight culture (in duplicates), the starting OD (at 600 nm) was measured and all the cultures were normalized to start at the same OD. An OD measurement was taken every hour for seven hours. As a control of the bacterial growth, a culture of untransformed *Salmonella* was also inoculated into plain LB medium. In two independent experiments, the clones A<sub>177</sub>I<sub>178</sub>, A<sub>177</sub>V<sub>178</sub>, A<sub>177</sub>L<sub>178</sub> and I<sub>177</sub> did not grow in M9 medium, therefore the glyphosate-resistance assays could not be performed on them.

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#### 6.1.7 ISOLATION AND PURIFICATION OF THE EXPRESSED PROTEIN FROM BACTERIAL CLONES

One milliliter of overnight culture of each of the bacterial clones is inoculated into 100 ml of liquid LB medium containing chloramphenicol. The cells were

allowed to grow at 37°C until they reached an OD of 0.5-0.7 (approximately 3 ½ hours). IPTG was then added to the cultures to a concentration of 1.0 mM. The cells were grown five additional hours. They were then pelleted at 4000 rpm for 20 minutes at 4°C.

The isolation and the purification of the His-tagged proteins were performed following the Qiagen Ni-NTA Protein Purification System. Cell lysates and eluates were run in duplicates on 12.5% acrylamide gels. One of the gels was silver-stained for immediate visualization, the second gel was transferred onto Millipore Immobilon-P membrane, and blocked overnight in 5% milk in TBS-T. The membrane was then exposed to Anti-His primary antibody solution (Amersham Pharmacia biotech, cat# 37-4710), followed by exposure to Anti-Mouse-IgG secondary antibody solution. (NIF825, from Amersham Pharmacia biotech ECL Western blotting analysis system, cat# RPN2108). Washes and detection reactions were performed according to the manufacturer instructions. Autoradiograms were developed after 5 minutes exposure.

## 6.2 RESULTS

Cells containing a mutation in the EPSPS gene produced cells that were both resistant to the herbicide glyphosate and that had a substantially similar growth rate in the absence or presence of glyphosate, as compared to the wild-type cells, irrespective of the presence of glyphosate (see Figure 6).

It was also demonstrated that the *Arabidopsis* clones containing a mutant EPSPS gene expressed the mutant protein at substantially the same level as the wild-type protein (see Figure 7).

The invention claimed and described herein is not to be limited in scope by the specific embodiments, including but not limited to the deposited microorganism embodiments, herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

## WE CLAIM:

1. A non-transgenic herbicide resistant plant, which plant expresses a mutant EPSPS gene product and which plant has substantially normal growth as compared to a  
5 plant expressing the wild-type EPSPS gene product.
2. A non-transgenic herbicide resistant plant, which plant expresses a mutant EPSPS gene product, which gene product has substantially the same level of catalytic activity as compared to the wild-type gene product.  
10
3. The plant according to claim 1 or 2 in which the herbicide is a member of the phosphonomethylglycine family.
4. The plant according to claim 3 in which the member of the  
15 phosphonomethylglycine family is glyphosate.
5. The plant according to claim 1 or 2 in which the EPSPS gene is mutated at one or more amino acid positions, said positions selected from the group consisting of Leu<sub>173</sub>, Gly<sub>177</sub>, Thr<sub>178</sub>, Ala<sub>179</sub>, Met<sub>180</sub>, Arg<sub>181</sub>, Pro<sub>182</sub>, Ser<sub>98</sub>, Ser<sub>255</sub> and Leu<sub>198</sub> in *Arabidopsis* or  
20 at an analogous amino acid residue in an EPSPS paralog.
6. The plant according to claim 5 in which the positions in the *Zea mays* paralog are selected from the group consisting of Leu<sub>97</sub>, Gly<sub>101</sub>, Thr<sub>102</sub>, Ala<sub>103</sub>, Met<sub>104</sub>, Arg<sub>105</sub>, Pro<sub>106</sub>, Ser<sub>23</sub>, Ser<sub>179</sub> and Leu<sub>122</sub>.  
25
7. The plant according to claim 5 in which the positions in the *Brassica napus* paralog are selected from the group consisting of Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>, Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>.
- 30 8. The plant according to claim 5 in which the positions in the *Petunia hybrida* are selected from the group consisting of Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>, Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>.
9. The plant according to claim 1 or 2 in which the plant is selected from the  
35 group consisting of corn, wheat, rice, barley, soybean, cotton, sugarbeet, oilseed rape,

canola, flax, sunflower, potato, tobacco, tomato, alfalfa, poplar, pine, eukalyptus, apple, lettuce, peas, lentils, grape and turf grasses.

10. The plant according to claim 5 in which the mutated gene results in one or more of the following amino acid substitutions in the EPSPS enzyme in comparison with the wild-type sequence:

- (i) Leu<sub>173</sub> - Phe
- (ii) Gly<sub>177</sub> - Ala or Ile
- (iii) Thr<sub>178</sub> - Ile or Val or Leu
- 10 (iv) Ala<sub>179</sub> - Gly
- (v) Met<sub>180</sub> - Cys
- (vi) Arg<sub>181</sub> - Leu or Ser
- (vii) Pro<sub>182</sub> - Leu or Ser
- (viii) Ser<sub>98</sub> - Asp
- 15 (ix) Ser<sub>255</sub> - Ala
- (x) Leu<sub>198</sub> - Lys.

11. The plant according to claim 6 in which the mutated gene results in one or more of the following amino acid substitutions in the EPSPS enzyme in comparison with the wild-type sequence:

- (i) Leu<sub>97</sub> - Phe
- (ii) Gly<sub>101</sub> - Ala or Ile
- (iii) Thr<sub>102</sub> - Ile or Val or Leu
- (iv) Ala<sub>103</sub> - Gly
- 25 (v) Met<sub>104</sub> - Cys
- (vi) Arg<sub>105</sub> - Leu or Ser
- (vii) Pro<sub>106</sub> - Leu or Ser
- (viii) Ser<sub>23</sub> - Asp
- (ix) Ser<sub>179</sub> - Ala
- 30 (x) Leu<sub>122</sub> - Lys.

12. The plant according to claim 7 in which the mutated gene results in one or more of the following amino acid substitutions in the EPSPS enzyme in comparison with the wild-type sequence:

- 35 (i) Leu<sub>169</sub> - Phe
- (ii) Gly<sub>173</sub> - Ala or Ile

- (iii) Thr<sub>174</sub> - Ile or Val or Leu
- (iv) Ala<sub>175</sub> - Gly
- (v) Met<sub>176</sub> - Cys
- (vi) Arg<sub>177</sub> - Leu or Ser
- 5 (vii) Pro<sub>178</sub> - Leu or Ser
- (viii) Ser<sub>94</sub> -Asp
- (ix) Ser<sub>251</sub> -Ala
- (x) Leu<sub>194</sub> -Lys.

10 13. The plant according to claim 8 in which the mutated gene results in one or more of the following amino acid substitutions in the EPSPS enzyme in comparison with the wild-type sequence:

- (i) Leu<sub>169</sub> - Phe
- (ii) Gly<sub>173</sub> - Ala or Ile
- 15 (iii) Thr<sub>174</sub> - Ile or Val or Leu
- (iv) Ala<sub>175</sub> - Gly
- (v) Met<sub>176</sub> - Cys
- (vi) Arg<sub>177</sub> - Leu or Ser
- (vii) Pro<sub>178</sub> - Leu or Ser
- 20 (viii) Ser<sub>94</sub> -Asp
- (ix) Ser<sub>251</sub> -Ala
- (x) Leu<sub>194</sub> -Lys.

25 14. A method for producing a non-transgenic herbicide resistant or tolerant plant comprising

- a. introducing into a plant cell a recombinagenic oligonucleobase to produce a mutant EPSPS gene; and
- b. identifying a cell having a mutated EPSPS gene, which cell has substantially normal growth as compared to a corresponding wild-type plant cell.

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15. A method for producing a non-transgenic herbicide resistant or tolerant plant comprising

- a. introducing into a plant cell a recombinagenic oligonucleobase to produce a mutant EPSPS gene; and

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b. identifying a cell having a mutated EPSPS gene, which encoded mutant EPSPS protein has substantially the same catalytic activity as compared to a corresponding wild-type EPSPS protein.

5                   16. The method according to claim 14 or 15 in which the recombinagenic oligonucleobase is a mixed duplex nucleotide or a SSMOV.

17. The method according to claim 16 in which the mixed duplex nucleotide contains a first homologous region which has a sequence identical to the sequence of at  
10 least 6 base pairs of the first fragment of the target EPSPS gene and a second homologous region which has a sequence identical to the sequence of at least 6 based pairs of a second fragment of the target EPSPS gene, and an intervening region which contains at least one nucleobase heterologous to the target EPSPS gene, which intervening region connects the first and second homologous region.

15                   18. The method according to claim 14 or 15 in which the recombinagenic oligonucleobase is introduced by electroporation.

19. The method according to claim 14 or 15 which the mutant EPSPS gene  
20 is mutated at one or more amino acid positions, said positions selected from the group consisting of Leu<sub>173</sub>, Gly<sub>177</sub>, Thr<sub>178</sub>, Ala<sub>179</sub>, Met<sub>180</sub>, Arg<sub>181</sub>, Pro<sub>182</sub>, Ser<sub>98</sub>, Ser<sub>255</sub> and Leu<sub>198</sub> in *Arabidopsis* or at an analogous amino acid residue in an EPSPS paralog.

20. The plant according to claim 19 in which the positions in the *Zea mays*  
25 paralog are selected from the group consisting of Leu<sub>97</sub>, Gly<sub>101</sub>, Thr<sub>102</sub>, Ala<sub>103</sub>, Met<sub>104</sub>, Arg<sub>105</sub>, Pro<sub>106</sub>, Ser<sub>23</sub>, Ser<sub>179</sub> and Leu<sub>122</sub>.

21. The plant according to claim 19 in which the positions in the *Brassica napus* paralog are selected from the group consisting of Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>,  
30 Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>.

22. The plant according to claim 19 in which the positions in the *Petunia hybrida* are selected from the group consisting of Leu<sub>169</sub>, Gly<sub>173</sub>, Thr<sub>174</sub>, Ala<sub>175</sub>, Met<sub>176</sub>,  
Arg<sub>177</sub>, Pro<sub>178</sub>, Ser<sub>94</sub>, Ser<sub>251</sub> and Leu<sub>194</sub>.

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23. The plant according to claim 14 or 15 in which the plant is selected from the group consisting of corn, wheat, rice, barley, soybean, cotton, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tobacco, tomato, alfalfa, poplar, pine, eukalyptus, apple, lettuce, peas, lentils, grape, turf grasses and *Brassica* sp.

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24. An isolated mutant EPSPS protein comprising the amino acid sequence depicted in SEQ ID NO:2, in which amino acid position Leu<sub>173</sub> is replaced with Phe, Gly<sub>177</sub> is replaced with Ala or Ile, Thr<sub>178</sub> is replaced with Ile or Val or Leu, Ala<sub>179</sub> is replaced with Gly, Met<sub>180</sub> is replaced with Cys, Arg<sub>181</sub> is replaced with Leu or Ser, Pro<sub>182</sub> is replaced with  
10 Leu or Ser, Ser<sub>98</sub> is replaced with Asp, Ser<sub>255</sub> is replaced with Ala or Leu, Leu<sub>198</sub> is replaced with Lys, which mutant EPSPS protein has increased resistance or tolerance to a herbicide, which herbicide is a member of the phosphonomethylglycine family.

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1/10

DNA sequence:

cccttcacgtctttttagaaccacccattatctttcttagggcccaattgaaaaccacattttctttcacctaaccac  
ccaaagccttgacacatgttgacgtgaacaccaaactaacacgtgtcactgcccagtggttatgataaatgctcacc  
ataccagagtcataagagtttttggttggtgaaagatttgacggatgccttcttctcatttctcaccaactccctccaa  
cccaacaaaatgtttatattagcaaagccgccaagtgtaaacgaaagtttataaatttcatttctgtgatcttacgta  
attggaggaagatcaaaattttcaatccccattcttcgattgcttcaattgaagtttctcgg

[transit peptide start]

ATGGCGCAAGTTAGCAGAATCTGCAATGGTGTGAGAACCACATCTCTTATCTCCAATCTCTCGAAATCCAGTCAACGCA  
AATCTCCCTTATCGGTTTCTCTGAAGACGCAGCAGCATCCACGAGCTTATCCGATTTCTGTCGTCGTGGGGATTGAAGAA  
GAGTGGGATGACGTTAATTGGCTCTGAGCTTCGTCTCTTAAGGTCATGCTTCTGTTTCCACGGCGGAG

[mature peptide starts]

AAAGCGTCGGAGATTGTACTTCAACCCATTAGAGAAATCTCCGGTCTTATTAAGCTTCTGGCTCCAAGTCTCTATCAA  
ATCGGATCCTGCTTCTCGTGCTCTGTCTGAGGTATATCACTTCGTTTCGTCTTCTCTGTAATCTGAACCTTAGATT  
ATAAAGATTGATACTTTACCATTTTGCTGTGGTTTTATAGGGAACAACGTAGTGGACAACCTGTTGAATAGCGATGAC  
ATCAATTACATGCTTGATGCGTTGAAGAGATTGGGACTTAATGTGGAACTGACAGTGAAAATAATCGTGCTGTAGTTG  
AAGGATGTGGCGGGATATCCAGCTTCCATAGATTCAAAGAGTGATATCGAACCTTACCTCGGTAATGCAGGAACAGC  
AATGCGTCCACTTACCGCTGCGGTCACTGCTGCAGGTGGAACGCAAGGTAGATTGAAGGAGTTGATGCTTCTGGTAT  
TTGATGTTAAGGAATGGAGCTTTTGTGATGCTTTATGATCCATTTATCCAGTTATGTGCTTGATGGGGTGCCTCGT  
ATGAGAGAAAGACCTATAGGGGATTTGGTTGTTGGTCTTAAGCAGCTTGGTGCTGATGTTGAATGTACTCTTGAACTA  
ACTGCCCTCCTGTTCTGTGCAACGCTAATGGTGGCCTTCCCGGTGGAAGGTTAGATCTTGCAAATGGCATGTGAATAT  
GTAATCTCGTTCCCTACTCTATGAACACTTGCAGAAATGTGTGTTTCATCATAGCCTTAGCTTGACAAGATTTAGTTTT  
TAATCTACTCTCAACGGATGGATCCTAAAATAGAATCGGATTTGGTGATTGGTTTTCGTTCTCGATTACCGTTTTCTGT  
GTATGATTTCTTGATTAACAATTAGGAGACATGTTATGCATTTGCAGGTGAAGCTTTCTGGATCAATTAGTAGTCAGTA  
CTTGACTGCTCTGCTCATGTCTGCTCCCTTAGCTCTTGGAGACGTCGAGATTGAGATTGTGCGATAAATTAATTTCTGTT  
CCATATGTTGAAATGACATTGAAGTTGATGGAACGTTTCGGGGTTAGTGTGAGCATAGTGATAGCTGGGATCGTTTCT  
TTGTCAAGGGCGGGCAAAAATACAAGTAGGAGTTATTCTTTCTCTCTTTCTGAAATCACATCCCTTAGCTTGACAAT  
ATAATGACTAAAAGGTGAATGATTGAGGTCTCCGGTAATGCGTATGTAGAAGGTGATGCTTCTAGTGCATGTTATTTCT  
TTGGCTGGTGCTGCCATTACCGGTGAAACTGTCACAGTCGAAGGTTGTGGAACCTACCAGCTTGCAGGTAATATTTGTAC  
ACTGAATCATCGACGAGGCTGTTAAGTTTATAGTGAATTCGTCTAGGTCAAAGTTTCATCTTTTGACAAGTTGTATAT  
AACATATTCGCAAGATTCTAAGCTCAATTTTGTGATGAATCTCTAGGGAGATGTAAAATTCGCCGAGGTCTTGAGAA  
AATGGGATGTAAAGTGTCTGGACAGAGAACAGTGTGACTGTGACAGGACCACCTAGAGATGCTTTTGGAATGAGACAC  
TTGCGGGCTATTGATGTCAACATGAACAAAATGCCTGATGTAGCCATGACCCTTGGCGTCTGCTCTTTGCTGACG  
GTCCAACCACCATAGAGATGGTAAGTAAAAAGCTCTCTCTTATAATTAAGGTTTCTCAATATTCATGATCACTTAATT  
CTGTTTGGTTAATATAGTGGCTAGCTGGAGAGTAAGGAGACAGAAAGGATGATTGCCATTTGCACAGAGCTTAGAAAA  
GTAAGAGATTCTTATCTCTCTCTTCTGTCTCTTGACAGTGCTCATTCTAAGTAATTAGCTCATAAATTTGTGTGTTG  
TGTTGAGCTGGGAGCTACAGTGAAGAAGGTTGAGATTATTGTGTGATAACTCCGCCCAAAAAGGTGAAAACGGCAGAG  
ATTGATACATATGATGATCATAGAATGGCAATGGCATTCTCTCTTGACAGCTTGTGCTGATGTTCCAATCACCATCAACG  
ACTCTGGTTGCACAGGAAAACCTTCCCGACTACTTCAAGTACTTGAAAGAAACACAAAGCACTAAacaataaactc  
tgttttttcttctgatccaagctt

FIG. 1A

SUBSTITUTE SHEET (RULE 26)



2/10

**Protein sequence:**

MAQVSRICNGVQNPSLISNLSKSSQRKSPLSVSLKTQQHPRAYPISSSWGLKKSGMTLIGSELRPLKVMSSVSTAE  
KASEIVLQPIREISGLIKLPGSKSLSNRIILLAAALSEGTTVVDNLLNSDDINMYLDALKRLGLNVEDSENNAV  
EGCGGIFPASIDSKSDIELYLGNAGTAMRPLTAAVTAAGGNASYVLDGVPRMRERPIGDLVVGLKQLGADVECTLG  
TNCPPVRVNANGGLPGGKVKLSGSISSQYL TALLMSAPLALGDVEIEIVDKLISVPYVEMTLKLMEFVSVVEHSD  
SWDRFFVKGGQKYKSPGNAYVEGDASSACYFLAGAAITGETVTVEGCGTTSLOGDVKFAEVLEKMGCKVSWTENS  
TVTGPPRDAFGMRHLRAIDVNMNKMVDVAMTLAVVALFADGPTTIRDVASWRVKETERMIAICTELRKL  
GATVEEGSDYCVITPPKKVKTAEIDTYDDHRMAMAFSLAACADVPIITINDSGCTRKTFPDYFQVLERITKH

**FIG. 1B**

3/10

Arabidopsis thaliana wild type sequence:

Position	173	174	175	176	177	178	179	180	181	182	183
	L	G	N	A	G	T	A	M	R	P	L
	CTC	GGT	AAT	GCA	GGA	ACA	GCA	ATG	CGT	CCA	CTT

Arabidopsis thaliana mutant sequences:

Name	
A <sub>177</sub>	CTC GGT AAT GCA <b>GCA</b> ACA GCA ATG CGT CCA CTT L G N A A T A M R P L
I <sub>178</sub>	CTC GGT AAT GCA GCA <b>ATA</b> GCA ATG CGT CCA CTT L G N A G I A M R P L
A <sub>177</sub> I <sub>178</sub>	CTC GGT AAT GCA <b>GCA</b> <b>ATA</b> GCA ATG CGT CCA CTT L G N A A I A M R P L
I <sub>178</sub> S <sub>182</sub>	CTC GGT AAT GCA GGA <b>ATA</b> GCA ATG CGT <b>TCA</b> CTT L G N A G I A M R S L
A <sub>177</sub> S <sub>182</sub>	CTC GGT AAT GCA <b>GCA</b> <b>ACA</b> GCA ATG CGT <b>TCA</b> CTT L G N A A T A M R S L
A <sub>177</sub> I <sub>178</sub> S <sub>182</sub>	CTC GGT AAT GCA <b>GCA</b> <b>ATA</b> GCA ATG CGT <b>TCA</b> CTT L G N A A I A M R S L
V <sub>178</sub> S <sub>182</sub>	CTC GGT AAT GCA GGA <b>GTA</b> GCA ATG CGT <b>TCA</b> CTT L G N A G V A M R S L
L <sub>178</sub> S <sub>182</sub>	CTC GGT AAT GCA GGA <b>TTA</b> GCA ATG CGT <b>TCA</b> CTT L G N A G L A M R S L
A <sub>177</sub> V <sub>178</sub>	CTC GGT AAT GCA <b>GCA</b> <b>GTA</b> GCA ATG CGT CCA CTT L G N A A V A M R P L
A <sub>177</sub> L <sub>178</sub>	CTC GGT AAT GCA <b>GCA</b> <b>TTA</b> GCA ATG CGT CCA CTT L G N A A L A M R P L

FIG.2

4/10

	10	20	30	40	50	60	70	80	90	
1	ATGGCGAAGTTAGCAGAACTCGCAATGGTGTGCAGAACCCAT	---	CTCTATCTCCAATCTCTCGAAATCCAGTCAAGCAAAATCTCC	---	CTTATCGG					atepspcDNA . SEQ
1	ATGGCGCAATCTAGCAGAACTCGCAATGGTGTGCAGAACCCAT	---	CTCTATCTCCAATCTCTCGAAATCCAGTCAAGCAAAATCTCC	---	TTTCTCCG					bnepsdDNA . SEQ
1	ATGGCACAAATTAACAACATGGCTCAAGGATACAAGCCCTTA	---	ATCCCAATTCCAATTTCCATAAACCCCAAGTTCTTAATCTTCAAGTTTCTTG							petaroacDNA . SEQ
1	GC GG									zmepsps . SEQ
	100	110	120	130	140	150	160	170	180	190
95	TTTCT	---	CTGAAGCGCAGCAGCATCCACGAGCTTATCCGATTTCGTGCTGGGGATTGAAGAGAGTGGGATGACGTTAATGGCTCTGAGCTTCG							atepspcDNA . SEQ
98	TTCC	---	TTGAAGCGCATCAGC	---	CTCGAGCTT	---	CTTCGTGGGGATTGAAGAGAGTGGGATGACGTTAATGGCTCTGAGCTTCG			bnepsdDNA . SEQ
98	TTTTTGGATCTAAAAAACTGAAAAATTCAGCAATT	---	CTATGTTGGTTTTTGAAAAAAGATTCAATTTT	---	TATGCAAAAAGTTTTCG					petaroacDNA . SEQ
5										zmepsps . SEQ
	200	210	220	230	240	250	260	270	280	290
192	TCCTCTTAAGGTCATGCTCTGTTTCCAGCGCGGAGAAAGGTCGGAGATTGACTTCAACCCATTAGAGAAATCTCCGGTCTTATTAAGCTTCTTGCC									atepspcDNA . SEQ
180	CCCGTTAAGGTAACAGCTCTGTTTCCAGCTCCGAGAAAGCTTCAGAGATTGCTTCAACCAATCAGACAAATCTCGGGTCTCATTAAGCTACCCGGA									bnepsdDNA . SEQ
180	TTCCCTTAGGATTTAGCATCAGTGGCTAGACGACAGAGCCCTCTGAGATAGTGTGCAACCCATTAAAGAGATTTTCAAGCAGTGTAAATTTGCTTGGC									petaroacDNA . SEQ
14										zmepsps . SEQ
	300	310	320	330	340	350	360	370	380	390
292	TCCAAGTCTCTATCAAAATCGGATCCTGCTTCTCGGTCTCTGCTGAGGGAACAACCTAGTAGTGGACAACCTTGTTGAATAGCGATGACATCAATTACATGC									atepspcDNA . SEQ
280	TCCAATCTCTCCAATCGGATCCTCTTTCGGCTCTATCTGAGGGAACCTACTGTAGTGGACAACCTTGTTGAACAGTGAATGACATCAACTACATGC									bnepsdDNA . SEQ
280	TCTAAATCATTATCTAATAGAAATCTCTTTCGTGCTTATCTGAGGGAACAACCTGTTGTTGACAAATTTACTAAGTAGTGAATGATTTACATGATGC									petaroacDNA . SEQ
67	TCCAAGTGCCTTTCCAACCGGATCCTCTTACTCGCGGCCCTGTGCGAGGGGACAACAGTGGTTGATAACCTGCTGGAACAGTGAGGATGTCCACTACATGC									zmepsps . SEQ
	400	410	420	430	440	450	460	470	480	490
392	TTGATCGGTTGAAGAGATTGGGACTTAATGTGGAACTGACAGTGAATAATCTGCTGTAGTTGAAGGATGTGGCGGATATCCAGCTTCCATAGA									atepspcDNA . SEQ
380	TTGATCGGTTGAAGAGCTGGGGCTTAAGGTGGAACTGACAGTGAATAACCTGCGGTTGTTGAAGGATGCGGTGGAATAATCCAGCTTCTTTAGA									bnepsdDNA . SEQ
380	TTGGTGCCTTGAAAGACATTGGACTGCATGTAGAGGAAGATAGTGGCAACCAACGAGCTGTTGTTGAAGGTTGGTGGGCTTTTCCCTGTTGGTAAAGA									petaroacDNA . SEQ
167	TCGGGGCCCTTGAGGACTCTTGGTCTCTCTGTCGAAGCGGACAAAGCTGCCAAAAGAGCTGTAGTTGTGGCTGTGGTGAAGGATCCAGTTG									zmepsps . SEQ

FIG.3A

5/10

500	510	520	530	540	550	560	570	580	590	
TTCAAAGAGTGATCGAATTTACCTCGGTAAATGAGGAAGACCAATGCGTCCACTTACCGCTGCGGTGCTGTCAGGTGGAAACGCAAGTTATGTG										atepspsDNA. SEQ
492	TTCCAAGAGTGATATTGAGTTGTACCTTTGGGAAATGCAAGAACAGCCATGCGTCCACTCACCGCTGTCAGTTACAGCTGCAGTGGCAACGCGAGTTATGTA									bnepsdDNA. SEQ
480	GTCCAAGGAAGAAATTCAACTGTTCTTTGGAAATGCAAGAACAGCAATGCGGCACTAACAGCAGCAGTTACTGTAGCTGGTGGAAATTCAGGTATGTA									petaroacDNA. SEQ
264	TGCTAAAGAGGAAGTGCAGCTCTTCTTTGGGGAATGCTGGAATGCAATGCGGCAATGACAGCAGCTGTACTGCTGCTGGTGGAAATGCAACTTACGTG									zmepsps. SEQ
600	610	620	630	640	650	660	670	680	690	
CTTGATGGGGTGGCTCGTATGAGAGAAAGACCTATAGGGGATTTGGTTGTTGGTCTTAAGCAGCTTGGTCTGATGTTGAAATGTAATGTAATCTTTGGAACTAACT										atepspsDNA. SEQ
592	CTTGATGGGGTGGCTCGTATGAGAGAAAGACCTATAGGGGATTTGGTTGTTGGTCTTAAGCAGCTTGGTCTGATGTTGAAATGTAATGTAATCTTTGGAACTAACT									bnepsdDNA. SEQ
580	CTTGATCGAGTTCCTCGAATGAGAGAGAGACCAATTAGTGAATTTGGTTGATGGTCTTAACAGCTTGGTGCAGAGTTGATTTGTTCTTGGTACGAAAT									petaroacDNA. SEQ
364	CTTGATCGAGTTCCTCGAATGAGAGAGAGACCAATTAGTGAATTTGGTTGATGGTCTTAACAGCTTGGTGCAGAGTTGATTTGTTCTTGGTACGAAAT									zmepsps. SEQ
700	710	720	730	740	750	760	770	780	790	
GCCCTCCTGTTGTTGTCACGCTAATGGTGGCTTCCCGTGGAAAGGTGAAGCTTCTTGGATCAATTAGTAGTCAGTACTTGACTGCTGCTGCTCATGTC										atepspsDNA. SEQ
692	GCCCTCCTGTTGTTGTCACGCTAATGGTGGCTTCCCGTGGAAAGGTGAAGCTTCTTGGATCAATTAGTAGTCAGTACTTGACTGCTGCTGCTCATGTC									bnepsdDNA. SEQ
680	GTCCTCCTGTTGTTGTCACGCTAATGGTGGCTTCCCGTGGAAAGGTGAAGCTTCTTGGATCAATTAGTAGTCAGTACTTGACTGCTGCTGCTCATGTC									petaroacDNA. SEQ
464	GCCCACTGTTGTTGTCACGCTAATGGTGGCTTCCCGTGGAAAGGTGAAGCTTCTTGGATCAATTAGTAGTCAGTACTTGACTGCTGCTGCTCATGTC									zmepsps. SEQ
800	810	820	830	840	850	860	870	880	890	
TGCTCCCTTAGCTCTTGGAGACGTGGAGATTGAGATTGTCGATAAATTAATTTCTGTTCCATATGTTGAATGACATTGAAGTTGATGGAAAGTTTCGGG										atepspsDNA. SEQ
792	TGCTCCCTTAGCTCTTGGAGACGTGGAGATTGAGATTGTCGATAAATTAATTTCTGTTCCATATGTTGAATGACATTGAAGTTGATGGAAAGTTTCGGG									bnepsdDNA. SEQ
780	AGCTCCTTTAGCTCTTGGAGACGTGGAGATTGAGATTGTCGATAAATTAATTTCTGTTCCATATGTTGAATGACATTGAAGTTGATGGAAAGTTTCGGG									petaroacDNA. SEQ
564	AGCTCCTTTAGCTCTTGGAGACGTGGAGATTGAGATTGTCGATAAATTAATTTCTGTTCCATATGTTGAATGACATTGAAGTTGATGGAAAGTTTCGGG									zmepsps. SEQ
900	910	920	930	940	950	960	970	980	990	
GTTAGTGTGAGCATAGTATAGTGGGATCGTTTCTTGTCAAGGGCGGGCAAAAATACAAGTCTCCGGGTAATGCGGTATGTAGAAAGGTGATGCTTCTA										atepspsDNA. SEQ
892	GTTAGTGTGAGCATAGTATAGTGGGATCGTTTCTTGTCAAGGGCGGGCAAAAATACAAGTCTCCGGGTAATGCGGTATGTAGAAAGGTGATGCTTCTA									bnepsdDNA. SEQ
880	ATTTCTGTGGAGCACAGTAGTAGTGGGACAGGTTCTTTGTCCGAGAGGTGAGAAATACAAGTCTCCGGGTAATGCGGTATGTAGAAAGGTGATGCTTCTA									petaroacDNA. SEQ
564	ATTTCTGTGGAGCACAGTAGTAGTGGGACAGGTTCTTTGTCCGAGAGGTGAGAAATACAAGTCTCCGGGTAATGCGGTATGTAGAAAGGTGATGCTTCTA									zmepsps. SEQ

FIG.3B

**FIG. 3C**

	10	20	30	40	50	60	70	80	90	100											
1	MAQVSRICNGVQNP	SLISNLSKSRQKSP	LSVLKTKQHP	RAYPTSSMELKKSMTL	IGSELR	.....	PLKVMSSVSTA	EKASEIVLQPIRE	ISGLIKLPGSKLSLN	atepsps. PRO											
1	MAQSSRICHGVP	CVTISNLSKSNQKSP	FVSVLKTHQ	.....	PRASSMELKKSMTL	NGSVIR	.....	PVKVTASVST	EKASEIVLQPIRE	ISGLIKLPGSKLSLN	bnepsps. PRO										
1	MAQINNMAQSIQTL	NPNSNFHKPVQK	SSSFLVFGSKK	.....	LKNSA	.....	NSMLVLK	SDSIFMOKFC	SFRISASVATAQ	KPSEIVLQPIKEISGTVKLPGSKLSLN	petaraoa. PRO										
1	AG	.....	.....	.....	.....	.....	.....	AAEITVLQPIKE	ISGTVKLPGSKLSLN	zmpsps. PRO											
104	RILLAL	SEGTTVDNLLNSDD	INMYLDAL	KRLGLN	VEITSEN	NRVAV	EGCGGIF	PASIDS	KSDIELYLG	NAGTAMRPLTA	AVTAAGMASVVL	DGVP	MRERPT	ICDLV	atepsps. PRO						
100	RILLAL	SEGTTVDNLLNSDD	INMYLDAL	KKLGLN	VERDS	VNRVAV	EGCGGIF	PASDS	KSDIELYLG	NAGTAMRPLTA	AVTAAGMASVVL	DGVP	MRERPT	ICDLV	bnepsps. PRO						
100	RILLAL	SEGTTVDNLLNSDD	ITHMYLG	ALKTGL	HVEEDS	ANR	VAV	EGCGGL	FPVGESKEE	IQLFLG	NAGTAMRPLTA	AVTVAGENS	VVL	DGVP	MRERPT	ISDLV	petaraoa. PRO				
29	RILLAL	SEGTTVDNLLNSDD	EDVHMYLG	ALRTGL	SV	EADK	AAKRAV	VVVGCGG	FPV	EDAKEEV	QLFLG	NAGTAMRPLTA	AVTAAGMASVVL	DGVP	MRERPT	ICDLV	zmpsps. PRO				
214	VGLKQL	GADV	ECTLTG	NCPPVR	NANGGL	PGGKVK	LSG	ISSQVLT	ALLMSAP	LALGDVE	IEITVD	KLISVP	VVEMTLKLM	ERFGVSV	EHSSD	MDRFFVK	GQKYKSPGVA	atepsps. PRO			
210	VGLKQL	GADV	ECTLTG	NCPPVR	NANGGL	PGGKVK	LSG	ISSQVLT	ALLMAAP	LALGDVE	IEITVD	KLISVP	VVEMTLKLM	ERFGVSA	EHSSD	MDRFFVK	GQKYKSPGVA	bnepsps. PRO			
210	DGLKQL	GA	EVDF	LGTK	CPVR	IVTSKGL	PGGKVK	LSG	ISSQVLT	ALLMAAP	LALGDVE	IEITVD	KLISVP	VVEMTLKLM	ERFGVSI	EHSSD	MDRFFV	RGGQKYKSPGKA	petaraoa. PRO		
138	VGLKQL	GADV	DFLTG	DCPPVR	WNGIGL	PGGKVK	LSG	ISSQVLT	SALLMAAP	LALGDVE	IEITVD	KLISIP	VVEMTLRL	MERFGVKA	EHSSD	MDRFFYK	GQKYKSPKVA	zmpsps. PRO			
324	VVEGDASS	CVFLAG	AAITG	ETVVE	GCGTTS	SLQGD	VKFAE	VLEKMG	KVSWITENS	VTVTG	PPRDA	FGMRHLRA	IDVNMN	KMPDV	AMTLAV	VALFAD	GPTTIR	DVASM	MRV	atepsps. PRO	
320	VVEGDASS	CVFLAG	AAITG	ETVVE	GCGTTS	SLQGD	VKFAE	VLEKMG	KVSWITENS	VTVTG	PSRDA	FGMRHLRA	VDVNMN	KMPDV	AMTLAV	VALFAD	GPTTIR	DVASM	MRV	bnepsps. PRO	
320	FVEGDASS	CVFLAG	AAVTG	GTITV	VECGTNS	LQGD	VKFAE	VLEKMG	AEVITWENS	VTVKGP	PRSSSG	RHLRA	VDVNMN	KMPDV	AMTLAV	VALYAD	GPTTIR	DVASM	MRV	petaraoa. PRO	
248	VVEGDASS	CVFLAG	AAITG	GTITV	VECGTTS	SLQGD	VKFAE	VLEMMG	AKVITWETS	VTVTG	PPREP	FGRKH	KA	IDVNMN	KMPDV	AMTLAV	VALFAD	GPTTIR	DVASM	MRV	zmpsps. PRO
434	KETERM	IAICTEL	RKLGAT	VEEGSD	VCVITPP	KKVK	TAE	IDTYD	DHRMA	FAFSLA	ACAD	VPVITIND	SGCTR	KTF	PDYFQ	VLERIT	TKH	atepsps. PRO			
430	KETERM	IAICTEL	RKLGAT	VEEGSD	VCVITPP	AKVK	PAE	IDTYD	DHRMA	FAFSLA	ACAD	VPVITIND	PGCTR	KTF	PDYFQ	VLESIT	TKH	bnepsps. PRO			
430	KETERM	IAICTEL	RKLGAT	VEEGSD	VCVITPP	PEKLN	WTAIDTYD	DHRMA	FAFSLA	ACAD	VPVITIND	PGCTR	KTF	PNYF	DV	LQY	SKH	petaraoa. PRO			
358	KETERM	WAIRTEL	TKLGAS	VEEGSD	VCVITPP	PEKLN	WTAIDTYD	DHRMA	FAFSLA	ACAE	VPVITIRD	PGCTR	KTF	PDYF	DV	LSTF	VKN	zmpsps. PRO			

**FIG. 4**

8/10

<u>Oligo Name</u>	<u>Oligo Sequence (5'→3')</u>
ATEPS-A <sub>177</sub>	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGTGGACGCATTGCTGTT <b>GCT</b> GCATTACCGAG
ATEPS-AI	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGTGGACGCATTGCT <b>ATTGCT</b> GCATTACCGAG
ATEPS-IS	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGT <b>GAA</b> CGCATTGCT <b>ATT</b> CCTGCATTACCGAG
ATEPS-AS	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGT <b>GAA</b> CGCATTGCTGTT <b>GCT</b> GCATTACCGAG
ATEPS-AIS	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGT <b>GAA</b> CGCATTGCT <b>ATTGCT</b> GCATTACCGAG
ATEPS-I <sub>177</sub>	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGTGGACGCATTGCTGTT <b>ATT</b> GCATTACCGAG
ATEPS-VS	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGT <b>GAA</b> CGCATTGCT <b>ACT</b> CCTGCATTACCGAG
ATEPS-LS	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGT <b>GAA</b> CGCATTGCT <b>AAT</b> CCTGCATTACCGAG
ATEPS-AV	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGTGGACGCATTGCT <b>ACTGCT</b> GCATTACCGAG
ATEPS-AL	CGTTTCCACCTGCAGCAGTGACCGCAGCGGTAAGTGGACGCATTGCT <b>AATGCT</b> GCATTACCGAG

FIG.5

9/10

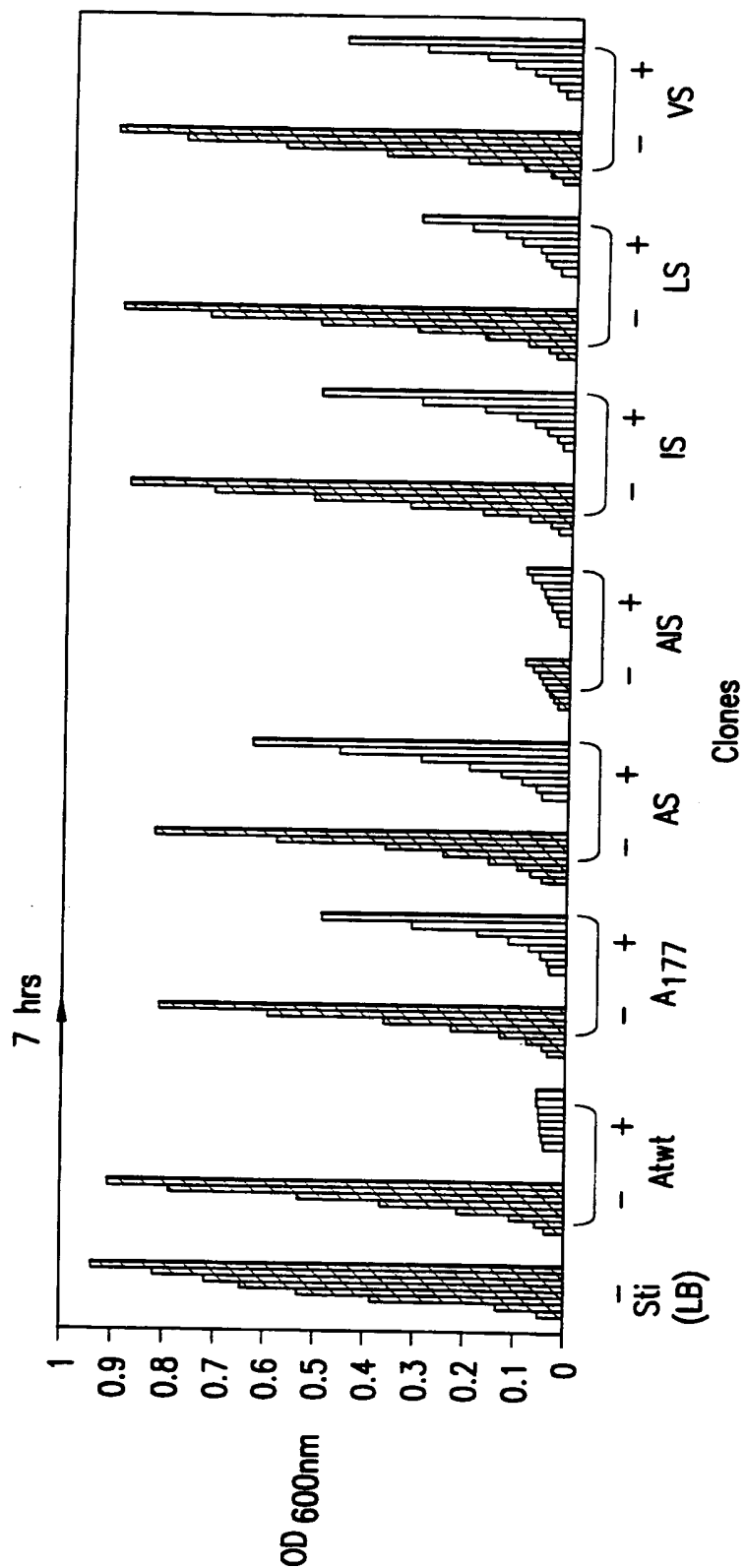


FIG.6



10/10

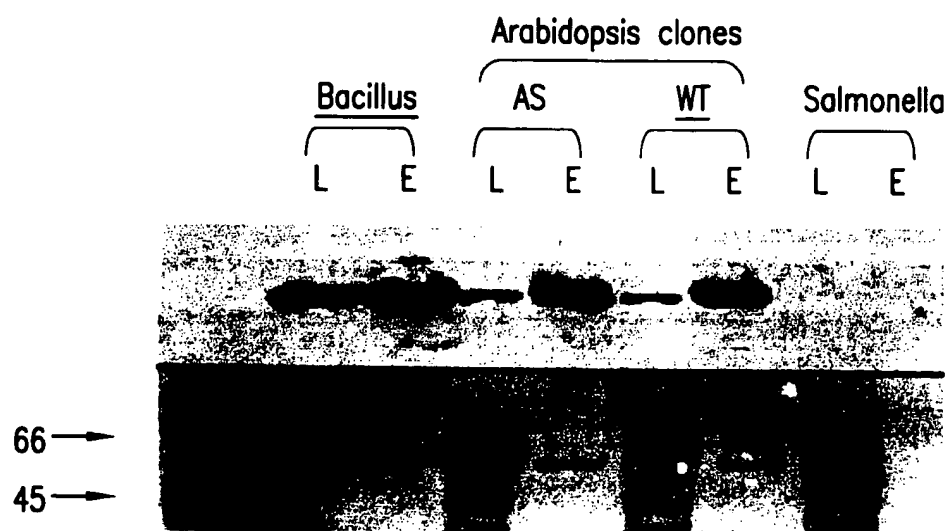


FIG. 7

## SEQUENCE LISTING

&lt;110&gt; VALIGEN(US), INC.

&lt;120&gt; NON-TRANSGENIC HERBICIDE RESISTANT PLANTS

&lt;130&gt; 7991-086-228

&lt;150&gt; 60/158,027

&lt;151&gt; 1999-10-07

&lt;150&gt; 60/173,564

&lt;151&gt; 1999-12-30

&lt;160&gt; 44

&lt;170&gt; FastSEQ for Windows Version 3.0

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&lt;211&gt; 2763

&lt;212&gt; DNA

&lt;213&gt; Arabidopsis thaliana

&lt;400&gt; 1

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Ser Leu Lys Thr Gln Gln His Pro Arg Ala Tyr Pro Ile Ser Ser Ser
35          40          45
Trp Gly Leu Lys Lys Ser Gly Met Thr Leu Ile Gly Ser Glu Leu Arg
50          55          60
Pro Leu Lys Val Met Ser Ser Val Ser Thr Ala Glu Lys Ala Ser Glu
65          70          75          80
Ile Val Leu Gln Pro Ile Arg Glu Ile Ser Gly Leu Ile Lys Leu Pro
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Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser
100         105         110
Glu Gly Thr Thr Val Val Asp Asn Leu Leu Asn Ser Asp Asp Ile Asn
115         120         125
Tyr Met Leu Asp Ala Leu Lys Arg Leu Gly Leu Asn Val Glu Thr Asp
130         135         140
Ser Glu Asn Asn Arg Ala Val Val Glu Gly Cys Gly Gly Ile Phe Pro
145         150         155         160
Ala Ser Ile Asp Ser Lys Ser Asp Ile Glu Leu Tyr Leu Gly Asn Ala
165         170         175
Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val Thr Ala Ala Gly Gly
180         185         190
Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg Met Arg Glu Arg Pro
195         200         205
Ile Gly Asp Leu Val Val Gly Leu Lys Gln Leu Gly Ala Asp Val Glu
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Cys Thr Leu Gly Thr Asn Cys Pro Pro Val Arg Val Asn Ala Asn Gly
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245         250         255
Tyr Leu Thr Ala Leu Leu Met Ser Ala Pro Leu Ala Leu Gly Asp Val
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Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val Pro Tyr Val Glu Met
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 325 330 335  
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 340 345 350  
 Thr Thr Ser Leu Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys  
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 Met Gly Cys Lys Val Ser Trp Thr Glu Asn Ser Val Thr Val Thr Gly  
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 Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala  
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 1               5               10

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&lt;210&gt; 24

&lt;211&gt; 1944

&lt;212&gt; DNA

<213> *Petunia hybrida*

&lt;400&gt; 24

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&lt;210&gt; 25

&lt;211&gt; 1335

&lt;212&gt; DNA

<213> *Zea mays*

&lt;400&gt; 25

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gggtgtgaaag	cagagcatte	tgatagctgg	gacagattct	acattaaggg	aggtcaaaaa	720
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<210> 26  
 <211> 516  
 <212> PRT  
 <213> Brassica napus

<400> 26

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Ile	Ile	Ser	Asn	Leu	Ser	Lys	Ser	Asn	Gln	Asn	Lys	Ser	Pro	Phe	Ser
			20					25					30		
Val	Ser	Leu	Lys	Thr	His	Gln	Pro	Arg	Ala	Ser	Ser	Trp	Gly	Leu	Lys
			35				40					45			
Lys	Ser	Gly	Thr	Met	Leu	Asn	Gly	Ser	Val	Ile	Arg	Pro	Val	Lys	Val
			50			55					60				
Thr	Ala	Ser	Val	Ser	Thr	Ser	Glu	Lys	Ala	Ser	Glu	Ile	Val	Leu	Gln
65					70					75				80	
Pro	Ile	Arg	Glu	Ile	Ser	Gly	Leu	Ile	Lys	Leu	Pro	Gly	Ser	Lys	Ser
			85					90						95	
Leu	Ser	Asn	Arg	Ile	Leu	Leu	Leu	Ala	Ala	Leu	Ser	Glu	Gly	Thr	Thr
			100					105					110		
Val	Val	Asp	Asn	Leu	Leu	Asn	Ser	Asp	Asp	Ile	Asn	Tyr	Met	Leu	Asp
			115			120					125				
Ala	Leu	Lys	Lys	Leu	Gly	Leu	Asn	Val	Glu	Arg	Asp	Ser	Val	Asn	Asn
			130			135					140				
Arg	Ala	Val	Val	Glu	Gly	Cys	Gly	Gly	Ile	Phe	Pro	Ala	Ser	Leu	Asp
145					150					155				160	
Ser	Lys	Ser	Asp	Ile	Glu	Leu	Tyr	Leu	Gly	Asn	Ala	Gly	Thr	Ala	Met
			165					170						175	
Arg	Pro	Leu	Thr	Ala	Ala	Val	Thr	Ala	Ala	Gly	Gly	Asn	Ala	Ser	Tyr
			180					185					190		
Val	Leu	Asp	Gly	Val	Pro	Arg	Met	Arg	Glu	Arg	Pro	Ile	Gly	Asp	Leu
			195			200						205			
Val	Val	Gly	Leu	Lys	Gln	Leu	Gly	Ala	Asp	Val	Glu	Cys	Thr	Leu	Gly
			210			215					220				
Thr	Asn	Cys	Pro	Pro	Val	Arg	Val	Asn	Ala	Asn	Gly	Gly	Leu	Pro	Gly
225					230					235				240	
Gly	Lys	Val	Lys	Leu	Ser	Gly	Ser	Ile	Ser	Ser	Gln	Tyr	Leu	Thr	Ala
			245					250						255	
Leu	Leu	Met	Ala	Ala	Pro	Leu	Ala	Leu	Gly	Asp	Val	Glu	Ile	Glu	Ile
			260					265					270		
Ile	Asp	Lys	Leu	Ile	Ser	Val	Pro	Tyr	Val	Glu	Met	Thr	Leu	Lys	Leu
			275			280						285			
Met	Glu	Arg	Phe	Gly	Val	Ser	Ala	Glu	His	Ser	Asp	Ser	Trp	Asp	Arg
			290			295					300				
Phe	Phe	Val	Lys	Gly	Gly	Gln	Lys	Tyr	Lys	Ser	Pro	Gly	Asn	Ala	Tyr
305					310					315				320	
Val	Glu	Gly	Asp	Ala	Ser	Ser	Ala	Ser	Tyr	Phe	Leu	Ala	Gly	Ala	Ala
			325					330					335		

Ile Thr Gly Glu Thr Val Thr Val Glu Gly Cys Gly Thr Thr Ser Leu  
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 Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Cys Lys  
                   355                  360                  365  
 Val Ser Trp Thr Glu Asn Ser Val Thr Val Thr Gly Pro Ser Arg Asp  
                   370                  375                  380  
 Ala Phe Gly Met Arg His Leu Arg Ala Val Asp Val Asn Met Asn Lys  
 385                  390                  395                  400  
 Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Phe Ala Asp  
                   405                  410                  415  
 Gly Pro Thr Thr Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr  
                   420                  425                  430  
 Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr  
                   435                  440                  445  
 Val Glu Glu Gly Ser Asp Tyr Cys Val Ile Thr Pro Pro Ala Lys Val  
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 Lys Pro Ala Glu Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala  
 465                  470                  475                  480  
 Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Lys Asp Pro  
                   485                  490                  495  
 Gly Cys Thr Arg Lys Thr Phe Pro Asp Tyr Phe Gln Val Leu Glu Ser  
                   500                  505                  510  
 Ile Thr Lys His  
                   515

&lt;210&gt; 27

&lt;211&gt; 516

&lt;212&gt; PRT

&lt;213&gt; Petunia hybrida

&lt;400&gt; 27.

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 Asn Ser Asn Phe His Lys Pro Gln Val Pro Lys Ser Ser Ser Phe Leu  
                   20                  25                  30  
 Val Phe Gly Ser Lys Lys Leu Lys Asn Ser Ala Asn Ser Met Leu Val  
                   35                  40                  45  
 Leu Lys Lys Asp Ser Ile Phe Met Gln Lys Phe Cys Ser Phe Arg Ile  
                   50                  55                  60  
 Ser Ala Ser Val Ala Thr Ala Gln Lys Pro Ser Glu Ile Val Leu Gln  
 65                  70                  75                  80  
 Pro Ile Lys Glu Ile Ser Gly Thr Val Lys Leu Pro Gly Ser Lys Ser  
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 Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr  
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 Val Val Asp Asn Leu Leu Ser Ser Asp Asp Ile His Tyr Met Leu Gly  
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 Ala Leu Lys Thr Leu Gly Leu His Val Glu Glu Asp Ser Ala Asn Gln  
                   130                  135                  140  
 Arg Ala Val Val Glu Gly Cys Gly Gly Leu Phe Pro Val Gly Lys Glu  
 145                  150                  155                  160  
 Ser Lys Glu Glu Ile Gln Leu Phe Leu Gly Asn Ala Gly Thr Ala Met  
                   165                  170                  175  
 Arg Pro Leu Thr Ala Ala Val Thr Val Ala Gly Gly Asn Ser Arg Tyr  
                   180                  185                  190  
 Val Leu Asp Gly Val Pro Arg Met Arg Glu Arg Pro Ile Ser Asp Leu  
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Val Asp Gly Leu Lys Gln Leu Gly Ala Glu Val Asp Cys Phe Leu Gly  
 210 215 220  
 Thr Lys Cys Pro Pro Val Arg Ile Val Ser Lys Gly Gly Leu Pro Gly  
 225 230 235 240  
 Gly Lys Val Lys Leu Ser Gly Ser Ile Ser Ser Gln Tyr Leu Thr Ala  
 245 250 255  
 Leu Leu Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile Glu Ile  
 260 265 270  
 Ile Asp Lys Leu Ile Ser Val Pro Tyr Val Glu Met Thr Leu Lys Leu  
 275 280 285  
 Met Glu Arg Phe Gly Ile Ser Val Glu His Ser Ser Ser Trp Asp Arg  
 290 295 300  
 Phe Phe Val Arg Gly Gly Gln Lys Tyr Lys Ser Pro Gly Lys Ala Phe  
 305 310 315 320  
 Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala  
 325 330 335  
 Val Thr Gly Gly Thr Ile Thr Val Glu Gly Cys Gly Thr Asn Ser Leu  
 340 345 350  
 Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Ala Glu  
 355 360 365  
 Val Thr Trp Thr Glu Asn Ser Val Thr Val Lys Gly Pro Pro Arg Ser  
 370 375 380  
 Ser Ser Gly Arg Lys His Leu Arg Ala Ile Asp Val Asn Met Asn Lys  
 385 390 395 400  
 Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Tyr Ala Asp  
 405 410 415  
 Gly Pro Thr Ala Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr.  
 420 425 430  
 Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr  
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 Val Glu Glu Gly Pro Asp Tyr Cys Ile Ile Thr Pro Pro Glu Lys Leu  
 450 455 460  
 Asn Val Thr Asp Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala  
 465 470 475 480  
 Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Asn Asp Pro  
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 Gly Cys Thr Arg Lys Thr Phe Pro Asn Tyr Phe Asp Val Leu Gln Gln  
 500 505 510  
 Tyr Ser Lys His  
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<210> 28  
 <211> 444  
 <212> PRT  
 <213> Zea mays

<400> 28  
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 35 40 45  
 Ser Glu Asp Val His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu  
 50 55 60  
 Ser Val Glu Ala Asp Lys Ala Ala Lys Arg Ala Val Val Val Gly Cys  
 65 70 75 80

Gly Gly Lys Phe Pro Val Glu Asp Ala Lys Glu Glu Val Gln Leu Phe  
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 Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val Thr  
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 Ala Ala Gly Gly Asn Ala Thr Tyr Val Leu Asp Gly Val Pro Arg Met  
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 Arg Glu Arg Pro Ile Gly Asp Leu Val Val Gly Leu Lys Gln Leu Gly  
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 Ala Asp Val Asp Cys Phe Leu Gly Thr Asp Cys Pro Pro Val Arg Val  
                             145                            150                            155                            160  
 Asn Gly Ile Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly Ser  
                             165                            170                            175  
 Ile Ser Ser Gln Tyr Leu Ser Ala Leu Met Ala Ala Pro Leu Ala  
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 Leu Gly Asp Val Glu Ile Glu Ile Asp Lys Leu Ile Ser Ile Pro  
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 Tyr Val Glu Met Thr Leu Arg Leu Met Glu Arg Phe Gly Val Lys Ala  
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 Tyr Lys Ser Pro Lys Asn Ala Tyr Val Glu Gly Asp Ala Ser Ser Ala  
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 Ser Tyr Phe Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Val Thr Val  
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 Glu Gly Cys Gly Thr Thr Ser Leu Gln Gly Asp Val Lys Phe Ala Glu  
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 Val Leu Glu Met Met Gly Ala Lys Val Thr Trp Thr Glu Thr Ser Val  
                             290                            295                            300  
 Thr Val Thr Gly Pro Pro Arg Glu Pro Phe Gly Arg Lys His Leu Lys  
                             305                            310                            315                            320  
 Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu  
                             325                            330                            335  
 Ala Val Val Ala Leu Phe Ala Asp Gly Pro Thr Ala Ile Arg Asp Val  
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 Glu Leu Thr Lys Leu Gly Ala Ser Val Glu Glu Gly Pro Asp Tyr Cys  
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 Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr Tyr  
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 Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Ala Glu  
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27941

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01H 1/06; C07H 21/04; C12N 5/04, 9/00, 15/01, 15/09, 15/29, 15/87  
US CL : 435/183, 410, 413, 418; 530/370; 536/23.1, 23.2; 800/276, 278, 300,300.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183, 410, 413, 418; 530/370; 536/23.1, 23.2; 800/276, 278, 300,300.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 6,066,786 ROSE-FRICKER) 23 May 2000 (23.05.00), entire reference.	1-4, 9
Y		5-8, 10-13
X	GORLANI et al. A glyphosate-resistant 5-enol-pyruvyl-shikimate-3-phosphate synthase confers tolerance to a maize cell line. Plant science 1992, Vol. 85, pages 9-15, entire reference.	1-4, 9
Y		5-8, 10-13
Y	PADGETTE et al. Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J. biol. chem. 25 November 1991, Vol. 266, No. 33, pages 22364-22369, especially Table 1, page 22365.	14-23
Y	US 5,756,325 A (KMIEC) 26 May 1998 (26.05.98), entire reference.	14-23
Y,P	US 6,004,804 A (KUMAR et al) 21 December 1999 (21.12.99), entire reference.	14-23

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

21 November 2000 (21.11.2000)

Date of mailing of the international search report

JAN 19 2001

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

David Kruse

Telephone No. 703-308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27941

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☒

- The additional search fees were accompanied by the applicant's protest.  
No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27941

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-23, drawn to a non-transgenic herbicide resistant plant that expresses a mutant EPSPS gene product and a method of producing said plant.

Group II, claim(s) 24, drawn to an isolated mutant EPSPS protein containing amino acid substitutions at specific sequence sites.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The isolated mutant EPSPS protein of Group II lacks a corresponding technical feature with the transgenic-herbicide resistant plant and the method of producing said plant of Group I. The isolated mutant EPSPS protein of Group II requires multiple amino acid substitution and the EPSPS expressed in the transgenic plant of Group I only requires one amino acid substitution.

#### Continuation of B. FIELDS SEARCHED Item 3:

- (1) EAST (USPAT, Derwent, JPO, EPO); natural glyphosate resistance, non-transgenic herbicide resistance, recombinagenic oligonucleobase, site directed mutation [in] plant(s).
- (2) STN (BIOSIS, AGRICOLA, EMBASE, CAPLUS); EPSPS Mutant/Mutation [in] plant(s), Natural glyphosate resistance.
- (3) Sequence Search of SEQ ID NO:2